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Expression of the Full-length Human Recombinant Keratinocyte Growth Factor in *Pichia pastoris*

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Abstract

Keratinocyte Growth Factor (KGF) is a paracrine-acting and epithelium-specific growth factor produced by cells of mesenchymal origin. Based on preclinical data, recombinant KGF plays a critical role in protecting and repairing of damaged epithelial tissues. Despite great efforts to express recombinant human KGF(rhKGF) in different organisms, attempts for finding appropriate protein expression system with the ability of producing a properly folded and processed KGF needs further investigation. *Pichia pastoris* has been used successfully and extensively for production of industrial enzymes and pharmaceutical proteins. Herein, we investigated the affect of pro-region- α -factor early deletion on production and secretion of rhKGF in *Pichia pastoris*. Initially, expression of human KGF induced in MCF-7 cell line treated with 1, 25-Dihydroxy vitamin D3. The coding sequence of full-length rhKGF₁₉₄ was then cloned into the yeast integrative expression vector, downstream of α -factor and was integrated into *P. pastoris* genome. KGF protein was expressed in *P. pastoris* x33 cells, using α -factor signal peptide for translocation of KGF to ER. An internal human signal peptide was also arranged after α -factor for early removal of the pro-region in ER. RT-PCR results demonstrated that KGF mRNA was expressed successfully after induction by methanol. Recombinant KGF protein expression was detected by Western blotting in cell lysats, but not in conditioned media. A molecular weight of 17 kD for rhKGF₁₉₄ indicates that the α -factor and internal human signal peptides had been removed in x33 cells. The results indicate that in the absence of pro-region- α -factor, the recombinant KGF protein was not efficiently processed and transported within the biosynthesis-secretory pathway. As KGF protein is an unstable growth factor and tend to aggregate because of some native properties, It seems that presence of a chaperon molecule fusion with KGF is necessary for efficient secretion of the recombinant protein.

Keywords: Keratinocyte growth factor, *Pichia pastoris*, Signal peptide

Introduction

Keratinocyte Growth Factor (KGF, also known as FGF-7) is a member of the fibroblast growth factor family that binds solely to fibroblast growth factor receptor 2b (FGFR2b) (Finch and Rubin, 2004). KGF is produced by mesenchymal cells, and promotes proliferation, DNA repair and migration of different types of epithelial cells. KGF has an important role in repair of injured epithelium and wound healing in various tissues and organs (Yen et al., 2014).

A recombinant truncated KGF, named as Palifermin (KepivanceTM), has been approved by U.S. Food and Drug Administration to reduce incidence, duration and severity of mucositis in patients suffering from hematologic malignancies undergoing high doses of

radiation and chemotherapy, before haematopoietic stem-cell transplantation (Finch and Rubin, 2006).

These therapeutic applications require large quantities of purified biologically active recombinant FGF7. Due to the low production level of rhKGF in various expression systems, and also the poor stability and the protein's tendency to aggregate rapidly, pharmaceutical applications of the growth factor have been limited (Huang et al., 2012; Chen et al., 1994; Feng et al., 2014; Xue et al., 2014). Attempts for finding an expression system with high production level and appropriate folding of the recombinant protein with simple and high quality purification method needs further investigation.

Due to the growth inhibitory effect of rhKGF in *E.*

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coli host cells, the yield of KGF production in this organism has been low or barely detectable, and the produced proteins sometimes aggregate as inclusion bodies. Palifermin (a commercial N-terminally truncated recombinant KGF protein) produced in *Escherichia coli* host, with a molecular weight of 16.3 kDa. Expression of KGF as a fusion protein with glutathione s-transferase (GST) in bacterial systems improved the yields of protein production. However; relative to the expression of FGF1, FGF2 and FGF10, the production level in bacteria was still low and needs further steps for purification of the overexpressed protein (Luo et al., 2004). Using molecular dynamics simulation of our GST-KGF fusion protein, it was indicated that GST act as a chaperone and interact with positively charged residues in heparin binding sites of the rhKGF and avoid conformational changes caused by repulsion of those positive amino acids, and subsequent aggregation of KGF (unpublished observations).

As *Pichia pastoris* is a notable eukaryotic expression system and is being used frequently for expression and production of different recombinant proteins, we decided to design and optimize a secretory expression system using this organism. The ability to secrete properly folded proteins into the culture media is one of the main advantages of *P. pastoris* as a protein producing host. However, level of secreted foreign proteins varies widely in yeast because of some roadblocks in specific steps in the secretory pathway (Ahmad et al., 2014; Daly and Hearn, 2005). Therefore, it is important to study different factors for improving secretion efficiency of KGF in large scale. One critical question is whether folding of recombinant KGF can be properly achieved in ER, or it needs a molecular chaperon for avoiding aggregation and ER-associated degradation (ERAD).

In this study, the coding sequence of human full-length KGF was cloned and stably expressed in *P. pastoris*. For answer the aforementioned question, we used thenative signal peptide of KGF as an internal second signal peptide, for early removal of pro-region- α -factor sequence. We then investigated the effects of this in the expression and secretion of KGF in *P. pastoris* x33.

Materials and Methods

Cell culturing and RNA extraction

The cell line originated from a breast adenocarcinoma (MCF-7) was obtained from Pasteur Institute of Iran. The cells were grown in RPMI 1640 medium (Invitrogen, UK) supplemented with 10% FBS (Invitrogen, UK), 100 U/mL

penicillin, 100 mg/mL streptomycin, and 25 ng/mL amphotericin B, at 37°C and 5% CO₂. For minimizing effects of exogenous growth factors, medium was replaced with medium containing 1.5% FBS, 24h before treatment. Cells were treated with 1, 25-dihydroxyl vitamin D₃ (Sigma, St. Louis, MO), at different concentration (150-300nM). 36 hours after treatment, cells were detached from the six-well plates using trypsin/EDTA solution, and total RNA was extracted according to the standard manual of TRIzol solution.

Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification and construction of expression vector

The specific primers for RT-PCR to amplify KGF (Gen Bank accession number NM_002009.3) were designed using Gene runner (version 3.02; Hastings Software), Oligo v 6.54, and PerlPrimer v1.1.16 softwares. RT-PCR was carried out using Revert AID cDNA synthesis kit (Fermentas, Canada) and KGF reverse specific primer (table 1), according to manufacturer's protocol. The fragment coding for the full-length KGF (194aa) was amplified using PCR technique. The sequences of the forward and reverse primers were shown in table 1. Forward and Reverse primers contained *XhoI* and *XbaI* restriction sites, respectively, that marked as underline letters. PCR conditions were perform as following: initial denaturation at 94°C for 2min, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; with a final extension at 72°C for 5 minutes. The PCR products were electrophoresed on 1% agarose gel and visualized with ethidium bromide staining and under UV light. The product was ligated into the TA vector and transformed in DH5 α *E. coli*. The authenticity of the PCR products was confirmed by DNA sequencing (Microgen, South Korea). The KGF coding sequence was then cloned into pPICZ α A expression vector, with the cloning sites of *XhoI* at upstream and *XbaI* at downstream.

P. pastoris transformation

Recombinant (pPICZ α A/KGF) and control (pPICZ α A) plasmids were linearized with *SacI* restriction enzyme and then purified by purification kit (Gene all, South Korea).

Electro-competent *P. pastoris* strain X-33 was prepared according to the manufacturer's recommendations (Invitrogen). 10 μ g of linearized plasmids were transformed in 80 μ l of electro-competent X-33, using a Bio-Rad gene-Pulser apparatus (1.5 KV voltage, 25 μ F capacitance, and 400 Ω resistance).

The cells were plated on YPDS plates containing 100 µg/ml zeocin and incubated at 30°C for 2-3 days. To verify the chromosomal integration of the plasmids, the genomic DNA of some of X-33 transformant colonies was analyzed by PCR. Single colonies were suspended in 20 µl H₂O, and after being frozen and thawed, were used for PCR as templates. AOX1 universal primers and KGF specific primers (table 1) were used in PCR reactions. The PCR conditions were as following conditions: initiation at 95°C for 5 minutes, amplification for 30 cycles with: denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

Table 1. Name and sequences of the primers used for cloning and PCR.

Primer name	sequence
KGF reverse specific primer	5'CACTTAAAGAAATCTCCCTGCTG-3'
KGF forward specific primer	5'-CATGAACACCCGAGCACTAC-3'
KGF cloning Forward	5'CCGCTCGAGAAAAGAATGCACA AATGGATACTG-3'
KGF cloning Reverse	5'-TGCTCTAGATTAA GTTA TTGCCATAGGAAGAAAGTG-3'
AOX1 forward primer	5'-GACTGGTTCCAATTGACAAGC-3'
AOX1 reverse primer	5'-GCAAATGGCATTCTGACATCC-3'

Expression of recombinant KGF in *P. pastoris*

Selected clones were inoculated in BMGY medium (2% peptone, 1% yeast extract, 1.34% yeast nitrogen base (YNB), 100mM potassium phosphate PH6.0, 4×10⁻⁵% biotin, and 1% glycerol) and grown at 30°C and 300rpm unit reaching OD₆₀₀ of 3. The cells were centrifuged at 3000×g for 10 minutes at 4°C and resuspended to an OD₆₀₀ of 1.5 in BMMY medium and were grown for 96h at 30°C. the methanol was added to a final concentration of 0.5% (v/v) every 24 hours to maintain induction. For analyzing secretion of recombinant protein, the media was concentrated about 10-fold using a 10 kDa molecular weight cut-off membrane in a stir-cell filtration unit (Amicon). For analyzing intracellular protein expression, cell lysis were prepared with acid-wash 0.5 mm glass beads (Sigma, St. Louis, MO), based on manufacture's instructions.

Western blot analysis

Protein concentration in the medium supernatants and cell lysis was quantified by Bradford protein

determinations. Equal amounts of protein (about 10 µg for medium supernatants and 25 µg for cell lysis) were loaded on 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). After blocking, the membrane was blotted with a polyclonal goat KGF antibody (C-19) (1:300 dilution, Santa Cruz biotechnology, Santa Cruz, CA). After 1 hour of washing in PBS-T, the membrane was incubated with the rabbit anti-goat IgG horseradish peroxidase-conjugated antibody (1:3000 dilution, Santa Cruz biotechnology, Santa Cruz, CA). After washing in PBS-T, the peroxidase activity was visualized with a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) on a X-ray film.

Results

Preparing human KGF coding sequence and constructing a pPICZαA/KGF expression plasmid

Based on previous reports, established epithelial tumor cell lines derived from a variety of tissue sources were not expressing KGF protein. Nevertheless, vitamin D3 induced KGF transcript and protein expression in the human breast cancer cell line MCF-7 (Finch & Rubin 2006). For this reason, MCF7 cell line was treated with different concentrations (150, 200, and 300 nM) of 1,25D3 for 36 hours. Total RNA was extracted and due to low expression of KGF mRNA, KGF reverse primer was used in cDNA synthesis, instead of oligodT primer. Using KGF forward and reverse specific primers, KGF cDNA containing 654 nucleotides was amplified by PCR technique. Results demonstrated that optimum concentration of 1,25D3 was 300 nM (Figure 1B). For producing the fragment containing the coding sequences for KGF protein (194 amino acids) as well as the restriction sites for *XhoI* and *XbaI* enzymes at 5' and 3' ends, respectively, the fragment was amplified using PCR and KGF cloning primers (Figure 1C). This segment was cloned into *XhoI* and *XbaI* restriction sites of pPICZαA vector. A schematic representation of rhKGF protein with 194 amino acid is shown in Figure 1A. Positive clones were selected and the authenticity of the recombinant plasmid was confirmed by DNA sequencing.

Integration of hKGF/pPicza construct into *P. Pastoris* genome

The recombinant plasmid, pPICZαA/KGF₁₉₄, was linearized with *SacI* restriction enzyme and was electroporated into the competent *P. pastoris* X-33.

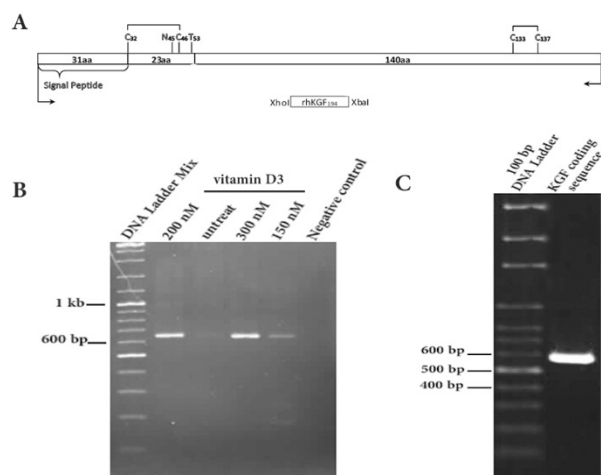


Figure 1. KGF coding sequence. Panel A. Schematic representation of rhKGF protein with 194 amino acid. RhKGF₁₉₄ contains a signal peptide (31 amino acid) and two disulfide bond linking Cys₃₂ to Cys₄₆ and Cys₁₃₃ to Cys₁₃₇, besides two N-linked and O-linked glycosylation sites on Asn₄₅ and Thr₅₃, respectively. Panel B. induction of KGF mRNA by treating MCF7 cell line with different concentrations of vitamin D3. Panel C. Amplification of the fragment coding KGF protein. Lane 2 amplicon Coding KGF₁₉₆ (608bp).

Integration of the DNA construct into the host genome was confirmed by PCR, using universal AOX1 and KGF specific primers. X-33 containing pPICZαA vector in its genome, was used as a negative control. The pattern of PCR product confirmed that both constructs were successfully integrated into the host X33 genome (Figure 2A). PCR with AOX1 primers resulted in amplification of two fragments of 1196bp (608bp+ 588bp) and 2.2Kb (AOX1 gene) for positive pPICZαA/full-length KGF transformant. On the other hand, the control transformants containing pPICZαA plasmid showed two fragments with the sizes of 588bp and 2.2Kb (lane 4 in Figure 2A). PCR with KGF specific primers amplified a 608bp fragment for pPICZαA/full-length KGF transformant. As it was expected, there was no amplification for control transformant (Lane 5 in Figure 2A).

Confirming human KGF mRNA expression in transformant *P. pastoris* cells

Using RT-PCR, KGF mRNA expression was investigated after 48 hours of induction by 1% methanol. Total RNA was extracted from induced and non-induced transformant expressing KGF. To avoid genomic's DNA amplification, DNase treatments were employed before cDNA synthesis, and no-RT reactions were used as negative controls. The result confirmed the expression of KGF mRNA in *P. pastoris* cells (Figure 2B).

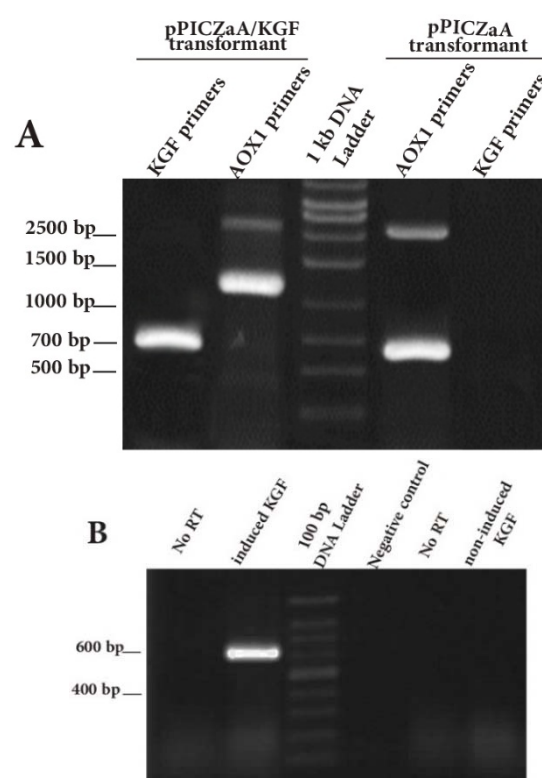


Figure 2. PCR analysis of *pichia* transformants. Panel A, PCR analysis for confirming the genomic integration of the expression construct. Lane 1 and 2: pPICZαA/full-length KGF transformant (using KGF special and AOX1 universal primers, respectively); Lane 3: DNA Ladder; Lane 4 and 5: pPICZαA transformant (using AOX1 universal and KGF special primers, respectively). Panel B, RT-PCR for detection of KGF expression at mRNA level. Lane 1 and 2: Methanol-induced transformant expressing full-length KGF recombinant protein and NoRT. Lane 3: 100 bp DNA Ladder. Lane 4: negative control. Lane 5 and 6: non-induced transformants expressing KGF recombinant protein and No RT.

Detection of human KGF protein expression in transformant *P. pastoris* cells

In order to examine KGF protein expression, Western blot analysis was performed using specific antibody against KGF. It was expected that the recombinant KGF protein to be secreted into the medium, because of the existence of signal peptide in KGF amino acid sequence. Therefore, concentrated media of the transformants were analyzed by Western blot technique. As it is shown in Figure 3A, no protein band was detected in media of different transformants expressing rhKGF₁₉₄. RhKGF₁₄₀ was used as a positive control with a molecular size of approximately 17 kD. On the other hand, media of transformants containing pPICZαA was used as a negative control, and no band was observed for pPICZαA transformant media.

In order to check if the recombinant protein is trapped inside the cell, the cell lysates were examined using Western blotting. As shown in figure 3B, the recombinant protein was detected in cell lysis of the transformants with a molecular size of 17 kD (Figure 3B). The results indicated that rhKGF₁₉₄ was not secreted into medium, but signal peptide and 23 N-terminal amino acids had been removed from the precursor protein.

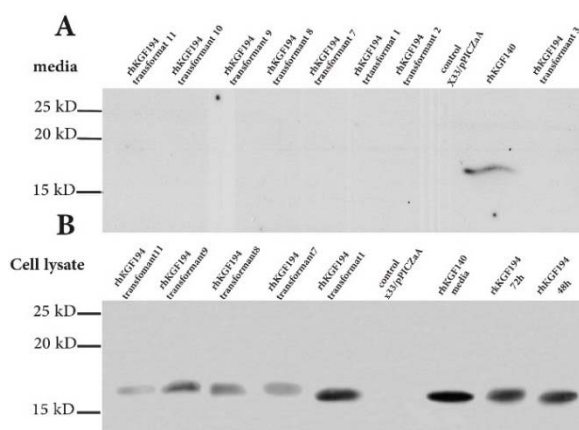


Figure 3. Western blot analysis for detection of recombinant proteins. Panel A. screening of different pPICZαA/ RhKGF₁₉₄ transformant media for detection of RhKGF₁₉₄. Media of pPICZαA/ RhKGF₁₄₀ transformant was used as a positive control. Media of pPICZαA transformant was used as a negative control. Panel B. screening of different pPICZαA/ RhKGF₁₉₄ transformant cells for detection of rhKGF₁₉₄ expression in cell lysis.

Discussion

One of the critical issues in biotechnological processes and drug production for human therapy is avoiding aggregation of recombinant protein during production. Because aggregation of the heterologous protein results in restricted solubility and biological activity and low yields of the products. Protein engineering strategies is a valuable approach to reduce aggregation and improve stability and folding of a protein drug during production (Vazquez et al., 2011). KGF has been proposed as a protein drug for treatment of pathologies associated with dermal adnexae, liver, lung, and gastrointestinal tract diseases, particularly wound healing in various tissues and organs (Finch et al., 2013; Yen et al., 2014). However, the production of the fairly unstable growth factor have been limited by its low level of expression in different expression systems. Therefore, it is of significant importance to design a more cost-effective and simpler expression system that improves the yield of recombinant KGF protein production with correctly folded and appropriately

post-translational modifications. In this study, *P. pastoris* expression system has been used for expression and secretion of a full-length human KGF protein containing yeast α -factor and native human signal sequences to elucidate some details of its biosynthesis and secretion in pichia expression system. This would open an avenue to improve the production yield and stability of the growth factor in yeast expression system.

Pichia pastoris expression system has been widely and successfully used to produce recombinant medical and industrial proteins. As *P. pastoris* combines the advantages of unicellular organisms (i.e. rapid growth and being easy to genetic manipulation) with the ability to produce proteins with eukaryotic post-translational modifications, the expression system is easier, faster and less expensive than other eukaryotic expression systems such as baculovirus or mammalian cell/tissue cultures (Ahmad et al., 2014; Daly and Hearn, 2005).

The most popular secretion signal that widely used in budding yeasts is α -factor signal peptide. The α -factor signal sequence contains pre- and pro-region. Pre-region is a 19-residue signal sequence and directs co-translational translocation of the recombinant protein into the ER. The pro-region contains 66 amino acids and is a hydrophobic peptide ended by short stretches of charged amino acids. Pro-region improves the efficiency of post-translational translocation across the ER membrane and is removed in the late-Golgi by the endo-protease kex2p (Ahmad et al., 2014; Fitzgerald and Glick, 2014). It is thought that the pro-peptide ensures proper folding of the nascent protein and can function as a molecular chaperon for secreted proteins. Some proteins can only be secreted in the presence of pro-peptide in the leader sequence (Lin-Cereghino et al., 2013). Based on previous reports and molecular dynamics simulation, protein engineering to make fusion protein of KGF with a chaperon molecule, like GST, is necessary for improving the stability of the protein and avoiding some conformational changes that proceed aggregation.

For these reasons, we used an internal second signal peptide to study the effect of early removal of pro-peptide in ER on the secretion of KGF protein. We hypothesized that the pro-peptide have a role as a chaperon for ER exit and secretion of KGF protein. In general, internal signal peptide could play two different roles; one role in directing translocation of the protein as a conventional signal peptide, and the other role in anchoring the protein on the membrane and creating a single or multipass integral protein.

Function of internal signal depends on the distance between the two signal peptides and post- or co-translational function of the first signal peptide (Coleman, Inukai et al. 1985). Since the internal signal peptide was removed in ER by signal peptidase, pro-region α -factor was cleaved in ER as it located before second signal sequence.

In **conclusion**, our data demonstrated that rhKGF₁₉₄ protein was expressed in the *P. pastoris* cell lysis but not secreted into the conditioned medium. Molecular weight of the produced recombinant KGF protein was estimated to be around 17 kDa. This molecular size suggested that the pre-pro- α factor, native human signal peptide, and its N-terminal 23-amino acids had been removed from rhKGF₁₉₄ protein. On the other hand, using different KGF constructs containing only a single N-terminal signal peptide did not interfere with the secretion of mature KGF (Bahadori et al., submitted). Therefore, we can conclude that pro-peptide- α -factor region in KGF expression construct has a vital role in proper production and secretion of KGF.

Acknowledgments

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Analysis of Chalcone Synthase and Chalcone Isomerase Gene Expression in Pigment Production Pathway at Different Flower Colors of *Petunia Hybrida*

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Abstract

Variegation in flower color is commonly observed in many plant species and also occurs on petunia (*Petunia hybrida*) as an ornamental plant. Variegated plants are highly valuable in the floricultural market. To gain a global perspective on genes differentially expressed in variegated petunia flowers, we investigated the expression of chalcone synthase (*chs*) and chalcone isomerase (*chi*) as two essential genes in biosynthesis pathway of pigment production. Also, we measured the concentration level of total flavonoids, naringenin chalcone and naringenin to evaluate the probably relationship between the expression profile of *chs* and *chi* genes and the concentration of mentioned pigments. The results indicated that chalcone synthase and chalcone isomerase expression had different profile in different petal color of *Petunia hybrida*. Because red flower color in petunia is related to the synthesis of pelargonidin-based (orange to red) pigments, our results suggest that the low chalcone synthase and chalcone isomerase expression levels in white petals reduce dihydrokaempferol formation, thereby inhibiting pelargonidin production. In contrast, the high expression levels of these genes observed in red petals ensure sufficient anthocyanin yields to make flowers red.

Keywords: Real-time PCR, *Chs*, *Chi*, *Petunia hybrida*

Introduction

Flower color is one of the most important characteristics in ornamental plant breeding. Flavonoids are the best characterized plant-specific secondary metabolites that accumulate in a broad range of plants, from gymnosperms to angiosperms (He et al., 2013). Flavonoids are natural products that contain a C6-C3-C6 carbon framework. They have a wide variety of biological functions including protection of cells against UV radiation, defense against pathogens and herbivores, regulation of auxin transport, and signaling between plant-microbe interactions, pollen growth and development, root nodule organogenesis, and most importantly, the flower colors facilitate attraction of pollinators and disperse the fruits and seeds. They are largely responsible for diverse pigmentation in the flowers, fruits, seeds, and leaves from shiny orange to pink, red, violet and blue (Kanazawa et al., 2007; Tan et al., 2013).

Flavonoids, a class of low-weight phenolic compounds, are derived from the general phenylpropanoids pathway. So far, more than 9,000 flavonoids have been identified. They are one of the

most important secondary metabolites that are classified into many subgroups such as chalcones, flavones, flavonols, flavandiols and anthocyanins according to the degree of oxidation and saturation of the central pyran ring (Liu et al., 2010; Tan et al., 2013).

Despite of the complexity of the flavonoid biosynthetic pathway, flower coloration is specifically connected to the flavonoid biosynthetic pathway (Liu et al., 2013; Tan et al., 2013). Therefore, investigation of differentially expressed genes from different-colored flowers and evaluating their relation to concentration of some pigments seems to be essential.

Among the genes and enzymes identified in the flavonoid pathway, the gene encoding the chalcone synthase enzyme (CHS) is the first dedicated one in this pathway, which catalyzes the stepwise condensation of three molecules of malonyl-CoA to one molecule of 4-coumaroyl-CoA to naringenin chalcone get synthesized, leading phenylpropanoids pathway to flavonoids biosynthesis (Figure 1). Production of chalcone starts with the transfer of a

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coumaroyl moiety from a p coumaroyl-CoA starter molecule to an active site cysteine (Cys164). Then, a series of condensation reactions of three acetate units derived from three malonyl-CoA molecules, each proceeding through an acetyl-CoA carbanion derived from malonyl-CoA decarboxylation, extends the polyketide intermediate. Following generation of the thioester-linked tetraketide, a regio-specific intramolecular claisen condensation forms a new ring system to yield chalcone (Yang et al., 2003; Dao et al., 2011).

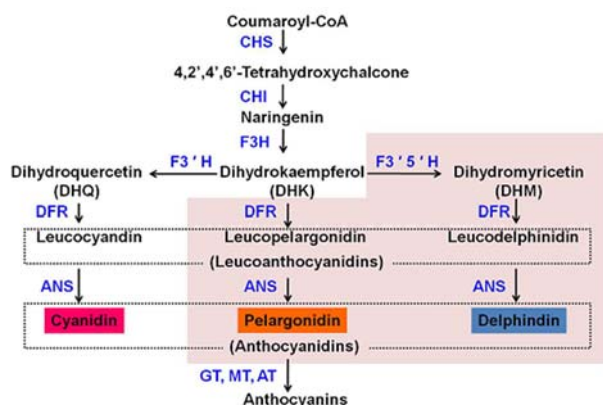


Figure 1. The flavonoid biosynthetic pathway leading to the synthesis of pigments (Adopted from He et al, 2013).

The CHS enzyme is also known as a type III of polyketide synthase enzymes (PKS) that is structurally and mechanistically the simplest PKS. These enzymes operate as homodimeric iterative PKS (monomer size of 42–45 kDa) with two independent active sites that catalyze a series of decarboxylation, condensation, and cyclization reactions (Deng et al., 2014).

The second key enzyme in flavonoid biosynthesis pathway is chalcone isomerase (CHI). This enzyme catalyzes the isomerization of naringenin chalcone into the corresponding flavanone (Figure 1) (Tunen et al., 1988). This enzyme belongs to the family of isomerases, specifically the class of intramolecular lyases. Chalcone isomerase has a core 2-layer alpha/beta structure. It has attracted much attention recently because of its involvement in the stress response and pigment production (Weely et al., 1983; Tunen et al., 1987). Petunias (*P. hybrida*) are one of the best annuals flowering plants for mass display in gardens and streets, and they also can be used for borders, containers, hanging baskets or as seasonal ground cover. They have a long flowering period, which can be from spring until frost occurs. Their flowers color range is large. It is much-loved, widely grown worldwide and plays an important role in improving the city environment (Wang et al.,

2006). Understanding mechanisms lead to pigmentation of flowers is the first step for manipulation the flower color that is desirable especially in ornamental plants.

To investigate and evaluate the probably relationship between the expression profile of *chs* and *chi* genes and the concentration of total flavonoids, naringenin chalcone (4,2',4',6'-Tetrahydroxychalcone) and naringenin, four distinct color (red, blue, pink and white) of *P. hybrida* were selected and assessed in present study.

Materials and Methods

Plant material

Potted plants of *P. hybrida* were grown under standard greenhouse conditions (16-17°C night temperature and 21-24°C day temperature and photoperiod 16/8 (light/dark)). Expanded white, pink, red and blue petals of *P. hybrida* were separately collected (Figure 2) and immediately immersed in liquid nitrogen after excision and preserved in a -80°C ultra-low temperature freezer until RNA extraction. Simultaneously, the same petal tissues were gathered to measure the mentioned pigments content.



Figure 2. The commercial varieties of *Petunia hybrida* with different color of petals. From left to right: red, blue, pink and white petunia.

RNA extraction and cDNA synthesis

Total RNA was extracted separately from four colors of petunia (white, pink, red and blue) using Denazist Column RNA Isolation Kit (#S-1020, Iran). RNA integrity was confirmed by 1% agarose gel electrophoresis. After treating with DNase I (Thermo Scientific #EN0525, USA) at 37°C for 30 min to remove probable DNA residues, RNA concentration was determined using a Nanodrop spectrophotometer. Synthesis of first strand cDNAs was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1622) following the manufacturer's protocol.

Quantitative real-time PCR

To perform the real-time quantitative PCR (qPCR), primers for the amplification of *chs* and *chi* genes were designed to amplify 188 and 137 bp fragments, respectively (Table1).

Table 1. Sequences of primers used for the amplification of *chs*, *chi* and *eflA* genes in *P. hybrida*.

Primers/Genes	chalcone synthase (<i>chs</i>)	chalcone isomerase (<i>chi</i>)	elongation factor (<i>eflA</i>)
Forward primer			
(5'→3')	AGACATAGTGGTGGTTGAAGTG	TCTCTCCAGTGTCGTTAC	TAAGTCTGTTGAGATGCACC
Reverse primer			
(5'→3')	TGAGCCTCTTGACCGATGG	ACAAACTTCCCTTCTATCTCCAG	CTGGCCAGGGTGGTTCATGAT

The qrtPCR was carried out with the SYBR® Premix Ex Taq™ II kit (TaKaRa #RR820L). Each reaction contained 2 µL of the first-strand cDNA as template, in a total volume of 20 µL reaction mixture. The amplification program was performed as 95°C/10min followed by 95°C/15sec, 60°C/15sec and 72°C/30sec (40 cycles).

In order to normalize the qPCR data, *elongation factor* (*eflA*) was selected as housekeeping gene and the following specific primers with product size of 180 bp were designed and used (Table 1).

The experiments were repeated three times on independently isolated mRNA preparation as biological repeats. To increase the reliability of gene expression analysis, real time PCR experiments were done with two identical technical replications. The accuracy of qrtPCR reactions were confirmed using melting curves for the products at the end of each run.

The calculation of relative gene expression was done based on methods that explain expression ratio equal to $2^{-\Delta\Delta C_t}$ (Pfaffl, 2001) while the white color flowers were employed as control samples.

Statistical Analysis

As mentioned above, our experiment was performed based on a completely randomized design with three (qrtPCR) and four (petal pigment concentration measurement) biological replications in the samplings. To increase the reliability of gene expression analysis, real time PCR experiments were also done with two identical technical replications. The statistically analyzes of the data were done using T-student and Tukey's range test ($\alpha = 0.05$; JMP v8).

Results

Gene expression profile in different flowers of *P. hybrida*

The expression profile of two genes, chalcone synthase (*chs*) and chalcone isomerase (*chi*) were investigated in four *P. hybrida* colors (white, blue, pink, and red) when petals were completely opened.

At this stage, the PCR products of all primers were coincided with bioinformatically predicted lengths. The highest level of *chs* expression was observed in red flowers petals with 4.6 times more than white flowers. In blue and pink flowers, the expression of *chs* gene had increased up to 4.1 and 3.3 times, in compare to the white color ones although there was not a statistically significant difference between these two colors together (Figure 3).

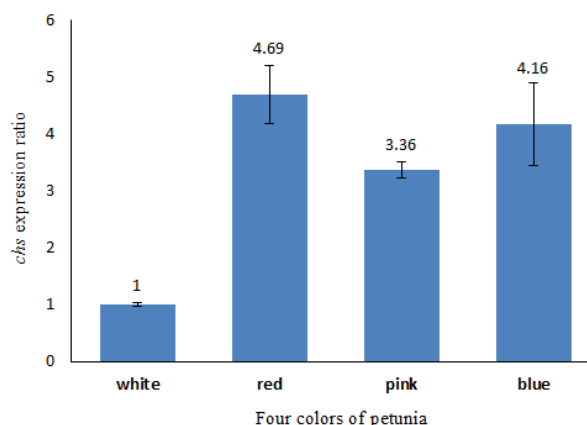


Figure 3. *Chs* gene expression in four colors of *P. hybrida*. Each data represents the average of three independent experiments. Error bars indicate the standard errors of the average of *chs* expression.

The analysis of *chi* expression in four *P. hybrida* also showed a significant expression increase of this gene in, red, blue and pink petals, compared to plants with white as control ones. *Chi* gene expression showed 3.27 times increase in plants with red flowers and 2.69 and 2.4 times increase in blue and pink flowers, relatively. However differences between blue and pink flowers were not statistically significant (Figure 4).

Measurement of total flavonoids, naringenin chalcone and naringenin

As was mentioned above, optical absorbance in 415 nm wavelength shows the level of total flavonoids in petal extract. Our results showed that

the absorbance of red, blue, pink, and white petals at this wavelength were 9.57, 5.95, 1.86, and 0.34, respectively (Figure 5).

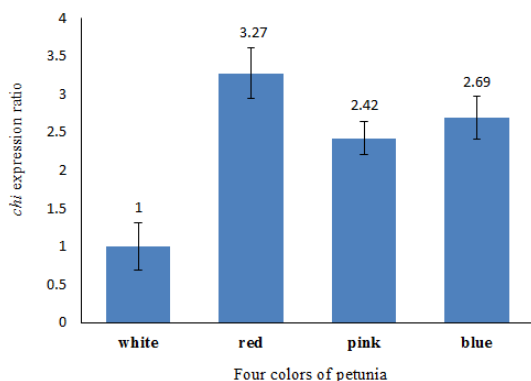


Figure 4. *Chi* gene expression in four colors of *P. hybrida*. Each data represents the average of three independent experiments. Error bars indicate the standard errors of the average of the *chi* expression.

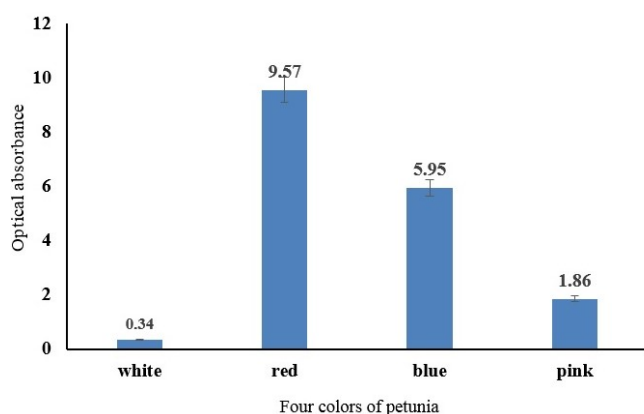


Figure 5. Optical absorbance of four *Petunia hybrida* in 415 nm that shows the level of total flavonoids. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the total flavonoid content.

Optical absorbance at 369 nm (related to naringenin chalcone concentration) showed an increase in petals with red, blue, and pink color in comparison with white ones. The highest optical absorbance at 369 nm was observed in red flowers (45.6), with 3.5 times more than white ones. The absorbance at 369 nm of blue and pink flowers was 30.3 and 15.2, respectively, however, the differences of pink and white petals were not statistically significant (Figure 6).

In order to estimate the relative concentration of naringenin, optical absorbance at 290 nm wavelength were measured in all four colors of petunias. Optical absorbances of red, blue and pink flowers were about 2.5, 1.6 and 1.07 times more than white flowers, while pink and white petal flowers showed

statistically equal absorbance at 290 nm (Figure 7).

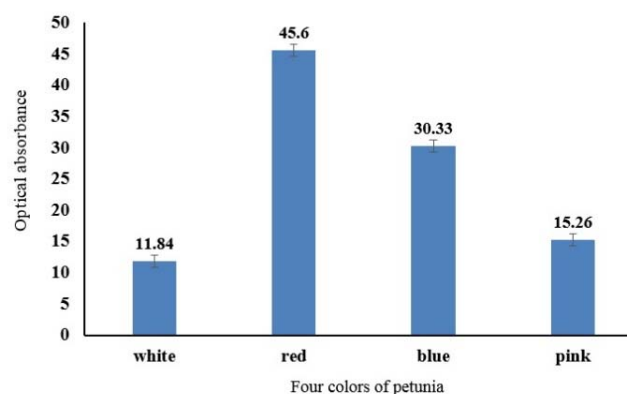


Figure 6. Optical absorbance of four *Petunia hybrida* in 369 nm that shows the level of naringenin chalcone. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the naringenin chalcone content.

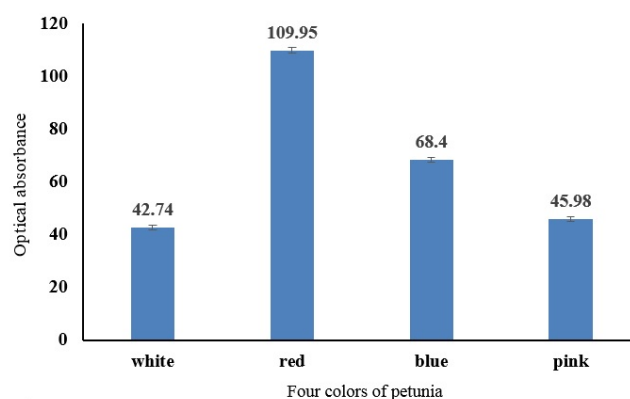


Figure 7. Optical absorbance of four *Petunia hybrida* in 290 nm that shows the level of naringenin. Each data represents the average of four independent experiments. Error bars indicate the standard errors of the average of the naringenin content.

Discussion

Flower color is largely determined by flavonoids pigments. Anthocyanins are a major colored class of flavonoids that are responsible for the pink, red, violet and blue colors of flowers and other tissues. Briefly, the anthocyanin biosynthesis pathway begins with the formation of chalcones by previously explained CHS. Then, CHI converts chalcone into naringenin. Naringenin is then hydroxylated at 3 positions of its central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK). DHK can then be further hydroxylated at the 3' position or at both the 3' and 5' positions of the B-ring to produce dihydroquercetin and dihydromyricetin, respectively (Figure 1). DHK, dihydroquercetin, and dihydromyricetin generally result in the production of brick-red/orang

pelargonidin-, red/pink cyanidin-, and blue/violet delphinidin-based pigments, respectively. Thus, the establishment of these three biosynthesis pathways is essential for diverse flower colors (Tan et al., 2013; Ma et al., 2014).

In this study, expression profile of *chs* and *chi* genes were studied at the completely open petals stage of all samples. The reason for choosing this step of flowering is completion of biosynthesis cycle for pigment production in each flower and appearance of their specific petal color.

Results of our experiments showed an expression increase in both *chs* and *chi* genes in plants with extreme flower colored ones (red and blue) in compared to other plants. The increase in optical absorbance at 369nm (naringenin chalcone) in red flowers can be attributed to higher expression of *chs* and presence of CHS (Figure 6). However, there was not any significant difference between *chs* expression of red and blue petal plants and *chi* expression of pink and blue ones. The different absorbance levels show different naringenin concentration in each petal color that can be attributed to more activities of chalcone isomerase enzyme.

Red flowers not only indicated the highest expression ratio of *chs* and *chi*, but also showed the highest concentration of total flavonoids, naringenin chalcone and naringenin. This pattern was not observed in pink and blue petal plants.

The reason for this might be attributed to two hypotheses. One is that red pigment production pathway in this plant is the main one compared to other pigments. As mentioned earlier and is shown in Figure 1, the pathway for red pigment production is the main pathway in anthocyanin production cycle, and other pathways that lead to blue and pink pigments are somehow derivation of this pathway by intermediate special enzymes. Therefore, upstream enzymes of this pathway (the pathway for red pigment production) like CHS, CHI and F3H must be more than other pathways to somehow provide these enzymes for other pigment production pathways like white and pink pigments.

The second hypothesis can be the common pathway for pigment production in plants with different flower colors. In other words, all petunia flowers with different colors have a common biosynthesis pathway to produce pigments, and only various physiological and environmental conditions cause activation of two flavonoid 3' hydroxylase (F3'H) and flavonoid 3', 5' hydroxylase (F3'5'H) enzymes during a certain time period that its outcome is production of blue and pink pigments. For example, one of the environmental factors that affects

anthocyanin biosynthesis pathway is pH of vacuole environment in a way that variations in pH, explicitly affect pigment production and special color. Research shows that in most plant species, acidic pH causes purple, violet and blue colors (Vlaming et al., 1983).

Regarding the concentration of these two compounds in red flowers, but not other colors, ecologic role of red color in absorbance of pollinators, can also be pointed out, since most insects and birds are attracted toward flowers with red and orange colors. In other words, ecologic and evolutionary evidences show the role of natural selection in development of most flowers with red, orange, and yellow colors that will result in appropriate interaction of these flowers with insects and birds (Rodríguez-Gironés & Santamaría, 2004; Rodríguez-Gironés & Santamaría, 2010). Therefore, more activities of these two enzymes in red flowers can be attributed to selection of these flowers in an evolutionary process, which leads to more activity of effective enzymes in anthocyanin production, especially enzymes in red pigment production.

According to the results of naringenin chalcone and naringenin level measurements, it can be concluded that the main pathway and other pigments such as blue and pink were derived from red pigment biosynthesis pathway.

These results coincide with reports of Koseki et al. (2005) that studied five main genes in petunia red star flowers and showed that expression of genes in red petals were increased compared to white petals. Griesbach et al. (2007) also compared white and red star petunia plants and observed that highest expression of *chs* gene was in red petals. In the study of *chs* and *chi* gene expression from white and red flowers of peach, a significant difference was observed between the two colors as red petals devoted the most *chs* gene expression to themselves. According to the conducted studies, it seems that CHS and CHI are two main enzymes in flavonoids/anthocyanins biosynthesis pathway and finally pigment production pathway; in a way that without the presence of these enzymes, biosynthesis cycle is not complete and consequently no pigment would be produced (Napoli et al., 1990; Mori et al., 2004).

However, these two enzymes are not ultimate enzymes and determiner of pigment type in plants with different flower color. In other words, although the presence of these two enzymes is essential for the initiation of pigment biosynthesis cycle in all studied plants, but their presence and concentration are not reliable indexes for predicting the final color of plant flowers.

As mentioned above, F3'H, F3'5'H and enzyme concentration of dihydroflavonol 4-reductase (DFR) can be pointed out as the main and effective enzymes in production of different colors resulted from anthocyanins biosynthesis pathway that produces pink, blue and red pigments, respectively (Pang et al., 2005; Sun et al., 2015).

Generally, final anthocyanin concentrations in plant cells are not determined solely by structural gene expression levels, and it is expected that some regulatory genes are obviously involved in the control of flavonoid biosynthesis. These regulatory genes, especially specific transcription factors, influence expression of many different structural genes that generally control pattern and intensity of anthocyanin biosynthesis. Up to now, three classes of TFs (bHLH, MYB and WD40) have been reported that seems to be related to flavonoid biosynthesis (Sun et al., 2015), however, further studies are needed to approve their importance in different variation. Our results uncover candidate genes associated with variegation in studied variety of petunia flowers. We also found that the higher expression of both *chi* and *chs* leads to higher concentration of studied pigments but the proportion of each one separately is not clear as was observed in blue and pink colored petals with different expression ratio of *chs* and *chi* in contrast to white and red colored flowers. Using reverse genetic may facilitate resolving this problem and providing unique insights into the molecular mechanisms controlling variegated flower pigmentation, and may eventually help the molecular engineering of variegated plants.

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The Effect of Caffeine on the Myelin Repair Following Experimental Demyelination Induction in the Adult Rat Hippocampus

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Abstract

Multiple sclerosis is characterized by the loss of oligodendrocytes and demyelination of axons. In this study, the effect of caffeine on spatial memory in rats was investigated following demyelination induction by lysolecithin (LPC). The expression of Myelin Basic Protein (MBP), Glial Fibrillary Acidic Protein (GFAP), and Olig2 (oligodendrocyte lineage marker) genes was also assessed in the hippocampus. Animals were divided into seven groups; control group: animals received normal saline by stereotaxic intrahippocampal injection in Dentate Gyrus (DG) area; LPC group: animals received 2 μ l lysolecithin by stereotaxic intrahippocampal injection in DG area (they were evaluated 7, 14, and 28 days after LPC injection; Caffeine- treated group: animals were treated with caffeine at doses of 30 mg/kg intraperitoneally for 7, 14, and 28 days after receiving LPC. Behavioral study was performed using Radial Arm Maze. Moreover, the RT-PCR was carried out for gene expression investigation. The demyelination and defective remyelination were noticeable on 28th day which suggests the demyelination decline caused by caffeine. Behavioral study showed that on the post-lesion days, the food finding time in the LPC group was significantly higher than that of the control group. Caffeine consumption significantly attenuated the food finding time in the treatment compared to the LPC group. The RT-PCR analysis indicated that the lysolecithin decreased the MBP expression especially on days 7 and 14 and conversely increased the Olig2 and GFAP expression. In addition, the caffeine enhanced the expression of MBP compared to that of the LPC group and reduced the Olig2 and GFAP expressions. Our results demonstrated that caffeine could increase the remyelination process in hippocampus and improve the spatial memory following demyelination induction by the LPC.

Keywords: Demyelination, Remyelination, Lysolecithin, Hippocampus, Caffeine

Introduction

Multiple sclerosis (MS) is a common autoimmune disorder of the central nervous system with a characteristic pathology that includes CNS demyelination and axonal damage, resulting in recurrent impairment of brain function (Keegan and Noseworthy, 2002). Lysophosphatidylcholines (LPC) also called lysolecithins, are a class of chemical compounds derived from phosphatidylcholins, which are toxic for the myelinating cells of CNS (Dixon et al., 1996). LPC has long been employed to create experimental demyelination by inducing myelin breakdown and apoptosis of Oligodendrocytes (Wallace et al., 2003). Indeed, lysophosphatidylcholine was recently shown to induce the influx of T cells with robust activation of macrophage/microglia in CNS. Activated microglial cells secrete a variety of cytokines which inhibit proliferation and differentiation of oligodendrocyte progenitors (Vela

et al., 2002). Caffeine is one of the members of methylxanthines which is largely anti-inflammatory in nature (Horrigan et al., 2006). As a result of having lipophilic property, caffeine easily passes from the blood brain barrier and as an adenosine receptors antagonist, it has different and complicated behavioral and biochemical effects on the CNS (Smith et al., 2003). Caffeine affects on neurogenesis, survival and proliferation of neural cells especially in hippocampus (Wentz and Magavi, 2009). The adult hippocampus, a vital center for learning and memory, is extremely vulnerable to various insults and neurological diseases (Nakafuku et al., 2002). Remyelination is a process in which myelin sheaths are generated and stored around the demyelinated axons. However, myelin sheaths are shorter and narrower (Franklin and French-Constant, 2008) and dependent on proliferation, migration, and differentiation of endogenous progenitor (Franklin, 2002).

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Although two major cell types, oligodendrocytes and neurons, are directly engaged in remyelination, it is clear that astrocytes and microglia are also involved in the myelin damage (Frederick and Miller, 2006 ; Zhang et al., 2001).

Astrocytes play an important role in pathological conditions of the nervous system. Accumulation of glial fibers is the histological landmark of the astrocyte response to CNS injury, appropriately named reactive gliosis. Such response is characterized by intense astrocyte proliferation (Bignami and Dahl, 1995).

Glial fibrillary acidic protein (GFAP) is an intermediate neurofilament expressed in the astrocytes and its levels were evaluated as a marker of astrocytes activity (McDonald and Ron, 1999). Oligodendrocytes are the myelinating cells of the CNS and are essential for proper brain function. These cells develop from an oligodendrocyte progenitor cells (OPC) (Raff et al., 1983) which arise in the subventricular zones and migrate extensively and differentiate into mature oligodendrocytes (Webb et al., 1995).

Olig2 (oligodendrocyte lineage marker) plays essential roles in oligodendrocyte specification and differentiation (Ligon et al., 2006) and is used in different studies as an oligodendrocyte precursor cells marker (Liu et al., 2007).

Myelin Basic Protein (MBP) is a major structural protein in myelin, thought to be primarily responsible for compaction and stabilization of the major dense line as well as playing a potent role in myelinogenesis (Jordan et al., 1989).

In the present study, a toxin-induced model of demyelination in the hippocampus of adult rat was used and the protective effect of caffeine on myelin repair and improvement of spatial memory following experimental demyelination by LPC was evaluated. Also, the gene expression for Olig2, MBP, and GFAP was assessed.

Materials and Methods

Animals

All experiments were carried out on adult male Wistar rats (Razi Institute, Karaj, Iran) weighting 180– 200 g (8–10 weeks).

Animals were housed four per cage under a 12-h light/dark cycle in a room with controlled temperature ($23 \pm 2^\circ\text{C}$).

Food and water were available ad libitum. All the experiments were carried out according to the protocol approved by the Animal Ethics Committee of Urmia University, Urmia, Iran.

Stereotaxic lysolecithin microinjection and Treatments

After 1 week of acclimatization, animals were deeply anaesthetized with a mixture of ketamine hydrochloride and xylazine (Sigma, Germany) (10 and 2 mg/kg intra-peritoneal., respectively) and positioned in rat stereotaxic instrument (Narishige, Japan) in a skull-flat situation. After shaving the corresponding skull surface, using a bladed scalpel, a midline incision was made at the middle of shaved site and then rats were cannulated. Guide cannuls was prepared from the dentistry needles- head 23G which was located bilaterally in Dentate Gyrus (DG) area of hippocampus according to stereotaxic atlas (Paxinos and Watson, 2007) with the coordinates of (AP= -2.8) toward the bregma, (L=+1.8) toward the middle line and (DV= +2.8) from the level of skull. The process of myelin destruction was carried out by injection of 2 microliter lysolecithin 1% (Sigma, St. Louis, USA) in saline 0.9% with the rate of 1 $\mu\text{l}/\text{min}$ in DG area of hippocampus (Dehghan et al., 2012; Mozafari et al., 2011).

Animals were divided into seven groups; control group: animals received normal saline by stereotaxic intrahippocampal injection in Dentate Gyrus (DG) area; LPC group: animals received 2 μl lysolecithin (LPC) by stereotaxic intrahippocampal injection in DG area (they were evaluated 7, 14, and 28 days after LPC injection; Caffeine- treated group: animals were treated with caffeine at doses of 30mg/kg intraperitoneally for 7, 14 and 28 days after receiving LPC.

Behavioral testing

At the first day to habituate the rats to the new environment of the radial arm maze (RAM), they were individually placed inside the center of the maze and were given 5 min to explore the maze. Behavioral testing was performed 1 hour after caffeine injection. During the habituation day rats received no food available and the arms the rats visited were recorded to ensure they were visiting all arms of the maze.

The second and third days of training sessions which was consists of two sessions per day for 5 minutes in the morning and in the evening. An arm entry was counted when all four limbs of the rat were within an arm. At the end of this 2 day training session, the rats reached the learning criterion of 90% (Tarbali et al., 2013, MCGurk et al., 1989). In all groups the time of finding food was evaluated. Behavioral study was carried out during 3 periods so that the first period included the days 1-7 post LPC injection, the second period encompassed the days 12-18 and finally the third period included the days 22-28 after LPC

injection.

Tissue Preparation and Histological Assessment

Animals were re-anesthetized on day 28 post lesion and were perfused intracardially with 0.1 M phosphate buffered saline (PBS) and then with a solution of 4% paraformaldehyde in 0.1 M PBS (pH=7.4). The hemispheres were taken out and post fixed overnight in the same fixative at 4 °C. For paraffin embedding, tissues were first dehydrated in alcohol, cleared by incubations in xylene, and finally embedded in paraffin for 3 h, and blocked. Coronal serial sections (5 µm thickness) were obtained from the hemispheres using a rotary microtome and then were stained with 0.1 % Luxol Fast Blue (British Drug House, UK) solution at 60 °C for 3 h. Adequate contrast was made by transient immersion of preparations in 0.05 % lithium carbonate and 70 % alcohol. After distilled water washes, the sections were counter stained with 0.1 % Cresyl Fast Violet (Merck, Germany) for 4 min. Sections were washed in distilled water again and dehydrated in a graded series of alcohols, then cleared in xylene, cover slipped and the sites of demyelination were verified (Khezri et al., 2013).

Gene Expression Study

For gene expression study, animals were divided into control and experimental groups. After LPC injection, treatment groups received 30 mg/kg caffeine for 7, 14 and 28 days. At the end of mentioned days, animals were killed and the hippocampus was extracted and immediately preserved in liquid nitrogen. Total RNA was isolated (Barres, 1991), using TRIZOL reagent (Sigma Aldrich) according to the manufacturer's instructions. The final total RNA pellet was suspended in 30 µl of DEPC (diethylpyrocarbonate) –treated water (Fermentas). 5 µl of total RNA was used for spectrophotometric determination of the RNA concentration at 260 nm. For each sample, cDNA synthesis was performed using 1 µg of total RNA, Oligo-dT primer, M-MuL V reverse transcriptase and RNase inhibitor (Fermentas) based on the manufacturer's instruction. PCR was performed using 1 µl synthesized cDNA as template, specific primers and 2X PCR Master Mix (Cinnagen, Tehran) based on the manufacturer's instruction. Primer sequences for Olig2, GFAP, MBP and β-actin, were designed on the basis of the published sequences in Gen Bank (Table 1). Segments of Olig2, GFAP, MBP, and β -actin (internal control) cDNAs were amplified for 32, 25, 26 and 25 cycles, respectively. The reaction parameters were adjusted to obtain a condition with

linear relation between the number of PCR cycles and PCR products and with linear relation between the initial amount of cDNA template and PCR product. Ten microliters of amplified products was run on 1.5% agarose gel (Roche, Germany). Agarose gels were stained by ethidium bromide (0.5 µg mL⁻¹) and visualized under a UV light. A 50 bp DNA ladder (Gene Ruler 50-1000 bp, Fermentas) was used as a molecular size marker. Semi-quantitative analysis of PCR products were done by band densitometry using a computerized image analyzing system (Carestream Gel Pro 212 Imager, USA).

Table 1. Sequences of primers used for RT-PCR amplification

Gene	Primer	Primers sequence
MBP	Forward	5'-CCCTCAGAGTCCGACGAGCT-3'
	Reverse	5'-GCACCCCTGTCACCGCTA-3'
Olig2	Forward	5'-CTCTTTGGCTGAACACTCCAA-3'
	Reverse	5'-CCCAAAAAGGATTCCGTTCCA-3'
GFAP	Forward	5'-CTCGTGTGGATCTGGAGAGGAA-3'
	Reverse	5'-GCCCTCCAGCAATTTCTGTAG-3'

Statistical Analysis

The results are expressed as mean ± SEM. Data from molecular assessments were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc using SPSS statistical software. The averages obtained from behavioral assessments were compared by using analysis of variance for repeated measures. For all analysis, $P < 0.05$ was considered significant.

Results

Myelin Staining

Histological evaluation of hippocampus by using myelin staining showed a normal staining for the control animals. Lysolecithin induced an obvious demyelination in rat hippocampus and this effect was noticeable. Twenty eight days after lysolecithin injection, some demyelination was detectable whereas in the rats treated by caffeine, myelin staining was rather improved. Representative micrographs are shown in

Figure 1.

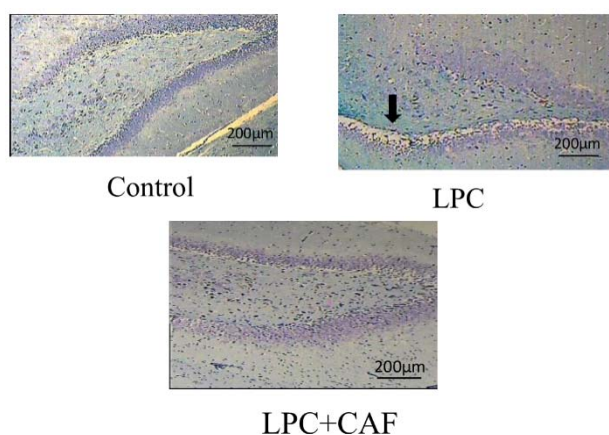


Figure 1. Representative micrographs showing the effect of LPC on demyelination and the effect of caffeine on myelin repair in the rat hippocampus DG area at day 28 post lysolecithin induced demyelination. LPC: lysolecithin-treated group, LPC+CAF: lysolecithin-treated animals injected with caffeine. Scale bar: 200 μ m.

Behavioral test

The results obtained from behavioral study during first period (i.e. the days of 1-7 after LPC injection) showed that the food finding time in LPC group was significantly ($P<0.01$, $P<0.001$) longer than control. Food finding time in treatment group was higher than control and in the days 5th – 7th there is significant difference ($P<0.001$), although, in the days 5th – 7th, the food finding time in treatment group was significantly ($P<0.05$, $P<0.01$) lower than LPC group (Figure 2A).

The time for food finding in second period (i.e. the days 12-18) in LPC group was significantly ($P<0.05$, $P<0.001$) longer than control group. Treatment with caffeine, caused significant ($P<0.05$, $P<0.01$, $P<0.001$) reduction of food finding time in radial maze compared to LPC group. Although during this period the time for food finding in (LPC+CAF) group was higher than control but at 16th to 18th days there is no significant difference (Figure 2B).

In third period the food finding time in LPC group gradually decreased, however, it was significantly ($P<0.01$) longer than control. Caffeine consumption in treatment group during 22-28 days, significantly ($P<0.01$, $P<0.001$) attenuated the time for finding food compared to LPC group. Also, despite the time in treatment group was higher than control, but there is no significant difference (Figure 2C).

Expression levels of Olig2, GFAP and MBP

To evaluate demyelination and remyelination following LPC injection in hippocampus, we accomplished several gene expression studies on the lesion site at 7, 14, and 28 days post lesion using

semi-quantitative RT-PCR. Gel documentations revealed bands with different densities for Olig2, MBP and Glial fibrillary acidic protein (GFAP) genes in the experiment time course.

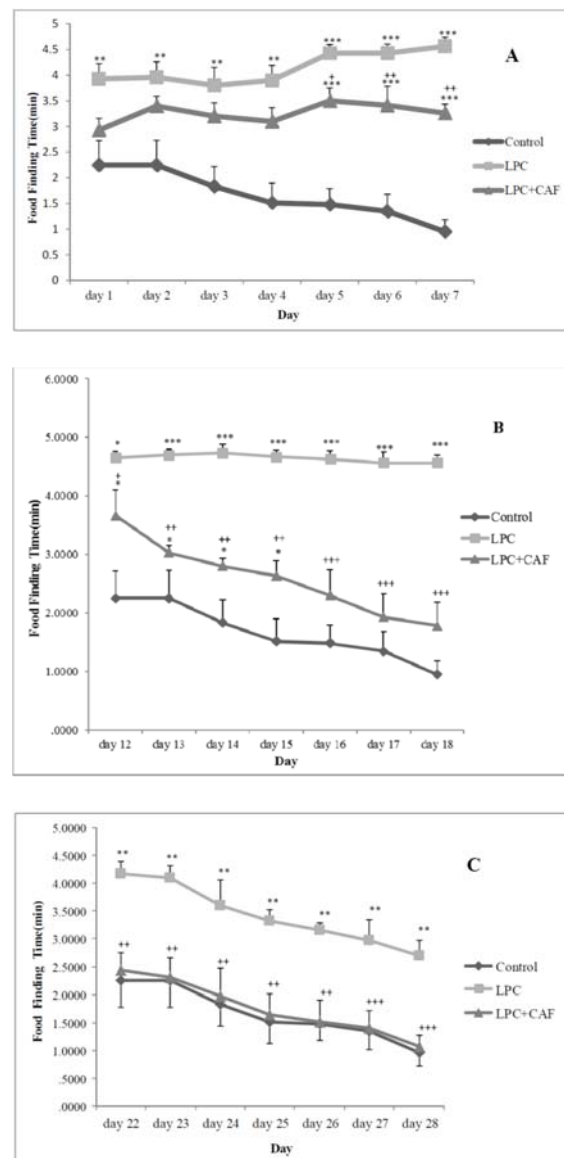


Figure 2. Impairment of hippocampal learning and memory following demyelination induction by LPC and caffeine injection in rats. (A) Food finding time at the first period (i.e. 1-7 day post lesion) of behavioral test was delayed in both LPC (Lysolecithin) and LPC+CAF (Lysolecithin + Caffeine) groups compared to control. However, time in LPC+CAF group was lower than LPC group. (B) In second period (i.e. 12-18 day post lesion), time in LPC group was higher than treatment and control. (C) In third period (i.e. 22-28 day post lesion), there was no noticeable difference between LPC+CAF group and control. Data were expressed as mean \pm SE. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to control, + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ compared to the LPC group ($n=8$).

Olig2 Gene Expression

Olig2 is used in different studies as an oligodendrocyte precursor cells marker (Liu et al., 2007). In the current study, Olig2 expression increased on 7 days post-lesion ($P<0.001$) and reached to the highest level on 14 days post-lesion ($P<0.001$) compared to the control group but it was decreased on the day 28 post-lesion, however, it still remained higher than control ($P<0.05$). The expression level of this gene in treatment group was significantly ($P<0.001$) lower than the LPC group all days post lesion (Figure 3A).

Glial fibrillary acidic protein (GFAP) Gene Expression

GFAP expression levels were evaluated as a marker of astrocytes activity (McDonald and Ron, 1999). The result of RT-PCR analysis showed that the lysolecithin significantly changed the GFAP expression level on days 7, 14 and 28 post lesion in patient groups to higher than control ($P<0.001$). This enhancement of GFAP level demonstrates astrocyte activity. Applying caffeine reduced the level of this gene in (LPC+CAF) group all days post lesion compared to LPC group ($P<0.01$, $P<0.001$) (Figure 3B).

Myelin Basic Protein (MBP) Gene Expression

MBP contributes to formation and compaction of myelin sheath (Baumann and Pham-Dinh 2001). The results showed that LPC injection caused significant reduction ($P<0.001$) in MBP gene expression on the day 7 and its mRNA level reached to minimum level on the day 14 post lesion in comparison to control ($P<0.001$). On the day 28, its expression was higher than days 7 and 14 but still it was significantly lower than control ($P<0.01$). These results signify hippocampus demyelination and myelinating cells depletion. Caffeine consumption significantly ($P<0.001$) increased MBP expression level in treatment groups compared to that of the LPC groups on the days 7, 14 and 28 post lesion. The day 28 had the highest level for MBP expression. Even on the day 28, its expression was significantly higher than control ($P<0.001$) (Figure 3C).

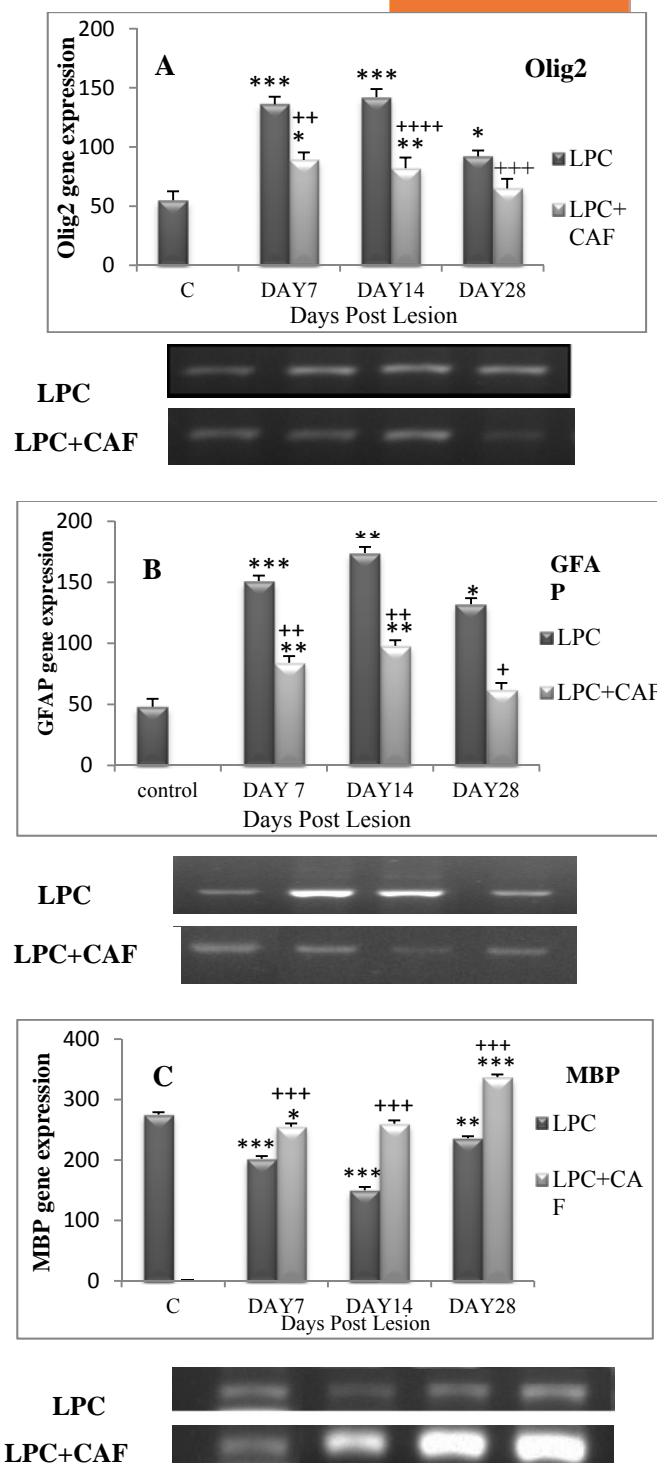


Figure 3. Assessment of gene expression in hippocampus. The gene expression level was assessed using RT-PCR and normalized to β -actin band density. (A) Changes in the expression of Olig2 as an oligodendrocyte precursor cell marker. (B) Changes in the GFAP expression following lesion induction in hippocampus. (C) Changes in the expression of myelin basic protein (MBP) gene following lysolecithin injection. * $P<0.05$, ** $P<0.01$ *** $P<0.001$ compared to control and + $P<0.05$, +++ $P<0.001$ compared to LPC group in the same day (n = 5). LPC (Lysolecithin) and LPC+CAF (Lysolecithin + Caffeine).

Discussion

Multiple sclerosis is one of the most common neurological disorders in young people with the age average of about 30 (Shivane and Chakrabarty, 2007). Inflammation and demyelination of CNS are its symptoms (Sherafat et al., 2012). Demyelination is the destruction of myelinating protein which forms a sheath around the axon of neurons. In the central nervous system, the myelin destruction process is occurred by the direct attack of immunity system to oligodendrocytes which from the myelin sheath and protect it, so, it can be a diagnostic aspect for MS (Tomassini and Pozzilli, 2009; Franklin and French-Constant, 2008).

Lysolecithin is an analogue of lysophosphatidylcholin and has a detergent effect with a special effect on myelinating cells. Hippocampus structure is known as one of the important gray substances which are affected by MS (Franklin and French-Constant, 2008). In this study, the effect of caffeine on memory recovery and also on remyelination was evaluated by expression measurement of Olig2, MBP and GFAP after lysolecithin intra hippocampus injection. Caffeine is one of the methylxanthin members which have anti-inflammatory property. In many neural destruction events it has been shown that the caffeine has neural protective effects (Franklin and French-Constant, 2008). This substance as adenosine receptor antagonist has many behavioral and biochemical effects in neural system (Wentz and Magavi, 2009).

The results obtained from our behavioral study at the first period showed that the time of finding food in LPC group was longer than control. However, all days of this period, the time for finding food in the caffeine-treated group was lower than LPC group. In second period, the food finding time in LPC group was longer than the first period and was also significantly ($P<0.05$, $P<0.001$) longer than control group. In these days, the LPC rats moved slowly in radial maze and they entered frequently in repetitive or empty of food arms. These cases show that the injection of lysolecithin in hippocampus led to demyelination in this area and also had the maximum destruction effect during these days. Thus, because of losing myelin sheath and the death of neurons and myelinating cells, the synaptic link between the neural cells in hippocampus encounters some problems and leads to memory and learning disorders. Makinodan and his colleagues (2008) showed that the lysolecithin led to special learning and memory disorder. They

suggested that after LPC injection, no maturity of oligodendrocytes was observed and it was indicative of the intensification of microglia and releasing the inflammatory factors and their provocative effects on the cell death of oligodendrocytes which led to delay in myelinating (Makinodan et al., 2008). The administration of caffeine in second period led to significant ($P<0.05$, $P<0.01$, $P<0.001$) decrease in the food finding time in treatment group compared to LPC group. Caffeine is known as a strong inhibitor of phosphodiesterase enzyme (Ribeiro and Sebastiao, 2010). Moreover, it leads to increase the level of cAMP by blocking the adenosine receptors. Cyclic AMP, by activating the route dependent on protein kinase A has anti-inflammatory effects and causes memory and learning reinforcement (Aandahl et al., 2002). In the third period, the time of finding food was decreased in the LPC group. Since no treatment was done in this group, this progressive reduction maybe due to the internal regeneration of hippocampus, of course, it had little effect on the improvement of memory disorder, because food finding time in this group was significantly ($P<0.01$) longer than the control and also the treatment groups. The time in the treated group with caffeine had no significant difference with control group. The rats treated with the caffeine were able to find the food in a shorter time and less errors. The findings showed that treatment with the caffeine has protective effects against the demyelination of neural cells and also causes to improve the spatial memory and the learning related to the hippocampus damage.

At the molecular level, the results showed that Olig2 gene expression in the LPC groups all days after LPC injection was significantly ($P<0.05$, $P<0.001$) higher than that of the control group. Olig2 belongs to a family of oligodendroglial transcription factors (Bignami and Dahl, 1995) and its expression is increased in reactivated oligodendrocyte precursor cells (Fancy et al., 2004). In our study, the observed Olig2 gene expression increase could reflect the existence of oligodendrogenesis potential in the demyelinated hippocampus. The expression level of this gene in treatment group was significantly ($P<0.001$) lower than LPC group all days post lesion and at day 28, there is no significant difference between control and treatment group. Dehghan and colleagues (2012) reported that the expression of Olig2 was increased on the days 7 and 13 post lesion, indicating immigration and activity of many oligodendrocyte precursor cells in demyelinating

area for generating the mature oligodendrocytes (Dehghan et al., 2012). Hence, lower expression of Olig2 in caffeine-treated groups implies more differentiation of precursor cells compared to LPC group.

GFAP levels are regulated under the pathological condition. The high regulation of GFAP is one of the important characteristics of astrocytes which is usually seen after the CNS lesion. Therefore, the study on GFAP regulation is useful for recognizing and evaluating the neurological and pathological problems (Gomes et al., 1999). The results of RT-PCR analysis suggest that the consumption of lysolecithin could increase the GFAP expression level on the days 7, 14, and 28 post lesion in the LPC group compared to that of the control group. The enhancement of GFAP level indicates astrocyte activity. Caffeine reduced the mRNA level of this gene in LPC+CAF group all days post lesion compared to the LPC group. The level of adenosine is increased in the CNS in pathological condition which in turn leads to enhancement of astrocyte activity and proliferation through the adenosine receptors and astrogliosis production through the metabolisms dependent on free radicals (Allaman et al., 2003). The long term effects of active astrogliosis lead to secreting the toxic and inflammatory factors and also bring about an inflammatory condition in the lesion area (Allaman et al., 2003; Pittock and Lucchinetti, 2007). Therefore, it is likely that the caffeine inhibits the adenosine activity under pathological condition by blocking the adenosine receptors especially A2A in the CNS and thus reduces the releasing of toxic and inflammatory factors which lead to the apoptosis of neural system. Mozafari and colleagues showed that the expression level of GFAP after the injection of lysolecithin in the optochiasma was maximum on the days 2th and 7th and it decreased on 28th day, although in this day, GFAP was higher than control. They reported that the astrocytes activity in damaged area is increased in response to the LPC induced demyelination and thus increased the GFAP expression (Mozafari et al., 2011).

MBP, a component of myelin proteins essential for myelin compaction and stability in CNS (Goudarzvand et al., 2010), is frequently used as an index of myelination (Goudarzvand et al., 2010; Messersmith et al., 2000). In the current study LPC injection caused to a significant reduction ($P<0.01$) in MBP expression all days post lesion compared to the control. Lowest expression level of MBP mRNA was observed on the day 14. These results demonstrate a hippocampus demyelination

and myelinating cells depletion. Afterwards, a significant MBP increase was observed on the day 28 that suggests an endogenous repair. The behavioral and histological observations in this study support this fact. The partial endogenous repair of myelin in this report might be due to recruitment, migration, and differentiation of adjacent OPCs or neural stem cells.

Caffeine consumption in treatment group significantly ($P<0.001$) increased MBP expression level compared to the LPC groups on the days 7, 14 and 28 post lesion. However, on the day 28 reached to the highest level compared to control. Perhaps newly differentiated oligodendrocytes express higher amount of MBP compared to older ones (Messersmith et al., 2000). As it was indicated in the behavioral test, the caffeine-treated group had the best function in the third period (i.e. the days 22 to 28) which reflects remyelination and a better learning in those days. The remyelination process requires the mature oligodendrocytes derived from the progenitor cells of the CNS which is located under the ventricular area. This observation may be due to accelerated process of OPCs (Oligodendrocyte Progenitor Cell) proliferation and migration towards the lesion (Menn et al., 2006; Dehghan et al., 2012) and also due to in advanced differentiation of OPCs to myelinating oligodendrocytes. OPCs have widespread distribution throughout the gray and white matter (Zhang et al., 1999; Setzu et al., 2004). The previous reports indicate caffeine can affect increase of proliferation, migration and differentiation of progenitor cells in hippocampus (Wentz and Magavi, 2009).

Conclusion

According to the obtained results, we can conclude that caffeine could attenuate the lysolecithin pathology at behavioral, histological, and molecular levels. Our findings may provide a neurological basis for the epidemiological investigation on the relation between caffeine consumption and the progression of MS in humans.

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Variations in Plasma Sex Steroid Hormones of the Wild Caspian Cyprinid Fish, Kutum (*Rutilus frisii kutum*)

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Abstract

Steroid hormones in plasma play an important role in reproductive cycles of animals especially during the final maturation stages. Steroid hormones synchronize gonad developments depending to fish species reproductive strategies. The wild Cyprinid fish, Kutum (*Rutilus frisii kutum*) is an ecologically and economically important fish species which inhabit in southern coastline of the Caspian Sea in Iran. Over the past few decades, natural reproduction of this species dramatically impaired due to the urbanization, civilization close to the land and shallow water in south western of the Caspian Sea. Therefore annual sex steroid hormones and gonads development were measured to assess the annual reproductive biology of female Kutum. In this study for the first time, our aims were to determine the annual variations in sex steroid hormones; 17 β Estradiol (E2), Progesterone (P) and testosterone (T) and gonad development of female Kutum. Our results showed that plasma steroid levels in females manifested in two phases in annual reproductive cycle; the resting phase (June - February) being characterized with the lowest level of steroid hormones and the peak reproduction activity phase (March–May) with simultaneously a significant increase in level of E2, P and T in plasma. Interestingly, comparing with other Teleost fish species the baseline level of E2 in plasma of Kutum during the resting phase to some extent was also huge. Increase in concentration of plasma E2 was in accordance with an increase of gonadosomatic index during spawning season. Our results contribute to our knowledge about the reproductive biology of Kutum and calls further long-term investigation.

Keywords: Sex steroid profile, Gonad development, Female Kutum

Introduction

Maturation and gonad development in animals are long processes that involving complex physiological and biochemical changes (Barannikova et al., 2002; Patiño and Sullivan, 2002). Steroid hormones in vertebrate animals have important role in gonad development and subsequently sexual maturity during annual reproduction cycles (Carragher and Pankhurst, 1993; Lubzens et al., 2010; Mojazi Amiri et al., 1996; Mylonas et al., 2010; Pankhurst and Carragher, 1992).

Sexual maturity and steroid hormones during reproductive cycles may change due to species characteristics such as age, sex and personality. Moreover, environmental stimuli can also play with role as drivers to change species steroid hormones levels in plasma. Many studies have shown seasonal changes of steroid hormone profiles and gonad developments in different fish species during spawning seasons (Matsuyama et al., 1988; Scott et al., 1984; Scott et al., 1983).

To explore spawning pattern in fish species it is crucial to understand annual reproductive cycle of plasma sex steroid hormones and gonad maturity (Lee and Yang, 2002; Manosroi et al., 2003). To our knowledge, there are few studies have been focused on annual sex steroid fluctuations in fish species (Chang and Yueh, 1990; Johnson et al., 1998; Sehafii, 2014). Moreover, yet there is only anecdotal and no well-detailed data of the annual fluctuations of sex steroid hormones in Caspian Sea Kutum.

Several studies on teleost fish species have investigated ovarian development, gametogenesis process and steroids hormone profiles such as white suckers, *Catostomus commersoni*; Cypriniformes, Teleostei (Scott et al., 1984; Stacey et al., 1984); rainbow trout, *Salmo gairdneri* (Fostier and Jalabert, 1986; Scott et al., 1983); goldfish, *Carassius auratus* (Kobayashi et al., 1989); orange roughy, *Hoplostethus atlanticus* (Pankhurst and Conroy,

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1988); gudgeon *Gobio gobio* (Rinchard and Kestemont, 1996); brown bullhead catfish, *Ictalurus nebulosus* (Rosenblum et al., 1987); Cyprinid fish Kutum, (Taghizadeh et al., 2013); vocal plainfin midshipman (Sisneros et al., 2004) and river catfish *Hemibagrus nemurus* (Adebisi et al., 2013). However, despite the importance of species reproductive biology during reproductive seasons surprisingly yet little is known about annual fluctuation of steroid hormones and gonad developments of teleost fishes in Caspian sea habitat. To our knowledge, there are a few studies focused on annual fluctuations and long term investigations on freshwater fish species (Chang and Yueh, 1990; Di Cosmo et al., 2001; Pavlidis et al., 2000; Scott et al., 1984) but not on Kutum in the Caspian sea habitat.

Cyprinid fish, Kutum, *Rutilus frisii* Kutum Kamenski 1091 is one of the endemic Teleost fish species which inhabit in the Caspian Sea and belongs to Cyprinidae family (Abdolhay et al., 2012; Abdoli, 1999; Bani and Vayghan, 2011). This species has economically and ecologically important values for biodiversity enrichment in the Caspian sea habitat. Kutum is an anadromous fish species and migrates from Caspian sea to adjacent rivers during spawning seasons (Abdolhay et al., 2011; Bani and Vayghan, 2011; Heidari et al., 2010). Males normally mature and are ready for spawning during third and fourth year and females mature and complete gonad development approximately in fourth year (Abdolhay et al., 2011). The spawning season of this species is from early March to late April with a peak spawning period in early April (Abdolhay et al., 2011; Keivany et al., 2012).

Since few decades ago, potential problems of pollution due to man-made activities have increased in shallow waters, estuaries which are relatively close to land. These locations are prone to human activities as well as attractive habitats to aquatic species. Due to population decline of this ecologically and economically important species, the Iranian fisheries organization (Shilat) started the artificial reproduction program in fisheries facilities (Abdolhay et al., 2011). To attain commercial fish species stock management, it is crucial to know more about annual sex steroid profile changes and gonad developments of bloodstock management for policy makers and fisheries companies to make more appropriate decisions for protecting riverside migratory routs managements and artificial reproduction procedures. This fact would be more pronounced especially if their population dynamics in natural environments are encountered with ecological and survival threat (Abdolhay et al., 2011;

Sehaffi, 2014). It is important for stakeholders, fisheries policy makers and reproduction facility managers to have more insights about annual reproductive cycle of this economically key fish species

In the current study our aims were to (a) quantify annual variations of the bioactive steroid hormones and (b) identify seasonal variations of gonad developments in Caspian Sea Kutum. We measured three main sex steroid hormone profiles, 17 β Estradiol (e2), Progesterone and Testosterone (T) which are closely related to female Kutum maturity and gonad development. Gonad development stages have been determined monthly according to the histological standard methods (Genten et al., 2009; Lubzens et al., 2010). We expected that temporal changes vary for sex steroid hormone profiles and are directly related to gonad developmental stages.

Materials and Methods

Experimental animals

Cyprinid fish individuals, *Rutilus frisii* Kutum were collected monthly from Sefid-Roud River estuary located in the south-west coast of the Caspian Sea (20 females per each month (20 \times 12=240, with total length : ~30–57 cm), (See figure 1). This range of size class is laid in the most female brood stocks which are used for artificial reproduction purposes in Iranian fisheries companies. All individuals were collected as much as possible in the same location. All fishes were packed in ice and transported to the laboratory facility center for further gonad development investigations. Age, body weight, gonad weight and sex were determined for each fish individual. biometrical data of all individuals such as total length (TL) and gonad weight were measured with accuracy of ± 5 mm. Quantitative indices with precision 1 mm, 0.01 g and 1 g, evaluation, registration forms and booklet were recorded. Scale samples were kept in the specific small-scale compartment for laboratory studies.

Steroid hormone assay

Plasma containing steroid hormones was collected to determine hormone concentration in female Kutum. Every first week of the month blood samplings were done for all individuals in the morning (approximately 8:00-11:00 AM). Five ml of blood was collected from the caudal vasculature using heparinized needle and samples carried in Eppendorf tubes were kept on ice boxes until centrifugation. Centrifugation was done at 6,000g for 5 min and then was transferred into 0.5 ml

Eppendorf tubes. Plasma samples were stored in the freezer at -80°C until steroid assay was performed.

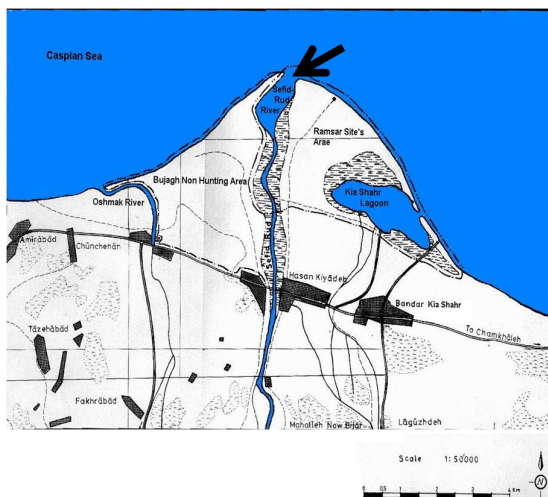


Figure1. The location of individual sampling in Sefid-rud river connected to Caspian sea, IRAN. The black arrow shows the place of sampling.

Plasma levels of the steroid hormones, E₂, P and T were measured by means of the radioimmunoassay (RIA) method after plasma extraction using the procedure described by (Kagawa et al., 1982; Rinchard et al., 1993).

Briefly, 50-100 μl of standards, controls and sample plasma was added into tubes coated by antibody (polyclonal rabbit antibodies were used). Thereafter, 500 μl of ¹²⁵I-labelled E₂ (radioactivity 170 kBq, Orion Diagnostica, Finland), ¹²⁵I-labelled Testosterone (Radioactivity 200 kBq, Orion Diagnostica, Finland), and 1 ml of ¹²⁵I-labelled Progesterone (Radioactivity 185 kBq, Immunotech, France) tracer was added to all tubes and incubated in a water bath (incubation times varied between steroid assays).

Following washes in phosphate buffer saline, radioactivity was counted using a gamma counter (Wallac/LKB gamma counter). The standard concentrations were ranged from 0-300 ng/ml for E₂, 0-14.4 ng/ml for T and 0-50 ng/ml for Progesterone.

Histological study

To evaluate the gonad development stages and changes among months, gonads has been dissected from fish body and kept in physiological serum solution. Gonads were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy (Poosti et al., 1996). Ovaries were classified by developmental stage

according to the relative abundance of the most advanced type of oocytes (Biswas, 1993; Kesteven, 1960; Rinchard and Kestemont, 1996). Five maturity stages were defined for females: stage I, ovogonial stage (presence of ovogonia, small oocytes and a few growing oocytes); stage II, primary oocytes (oocytes with homogenous cytoplasm, circular nucleus, and numerous nucleoli); stage III, cortical alveoli (oocytes with peripheral cortical alveoli, located nucleus, and several nucleoli); stage IV, vitellogenesis (increasing number and size of yolk granules); and stage V, final maturation (large hydrated oocytes with germinal vesicle near the cell membrane). Environmental factors, water temperature and salinity were also measured monthly. To evaluate the gonadosomatic index (GSI) gonads were dissected and weighted.

The gonadosomatic index was calculated as ovary weight divided by body weight multiplied by 100. The following formula was applied: $GSI = (GW/BW) \times 100$; Where GW= gonad weight (g) and BW= body weight (g),

Statistical analysis

One way analysis of variance (ANOVA) was applied for statistical analysis. To check for significant changes, Estradiol, Progesterone and Testosterone selected as dependent variables and temporal variation of month as an independent variable. When the ANOVA test was significant, analysis followed by Duncan's multiple range test (DMRT) to estimate the mean differences of different months per each hormone monthly variation and gonadosomatic index.

Spearman correlation test was done to check the correlation between sex steroid hormones with each other. Mean values were used to compare numerical data. Values are presented as mean \pm standard errors. Differences of $P \leq 0.05$ were considered statistically significant. All data were analyzed by using SPSS v. 21.05.

Results

Sex steroid measurements

Results showed that there is a significant effect of temporal variations on 17-beta estradiol levels (ng/ml) among months ($F_{11, 96}: 22.99, P < 0.001$) (See figure 2a). There was a significant increase for E₂ with onset of spawning season from February and the highest level of E₂ was observed in March. Post-hoc test, Duncan Multi comparison test results showed in the table 1. We did find a significant effect of months on level of plasma progesterone $F_{11, 96}: 14.73, P < 0.001$. (See figure 2b).

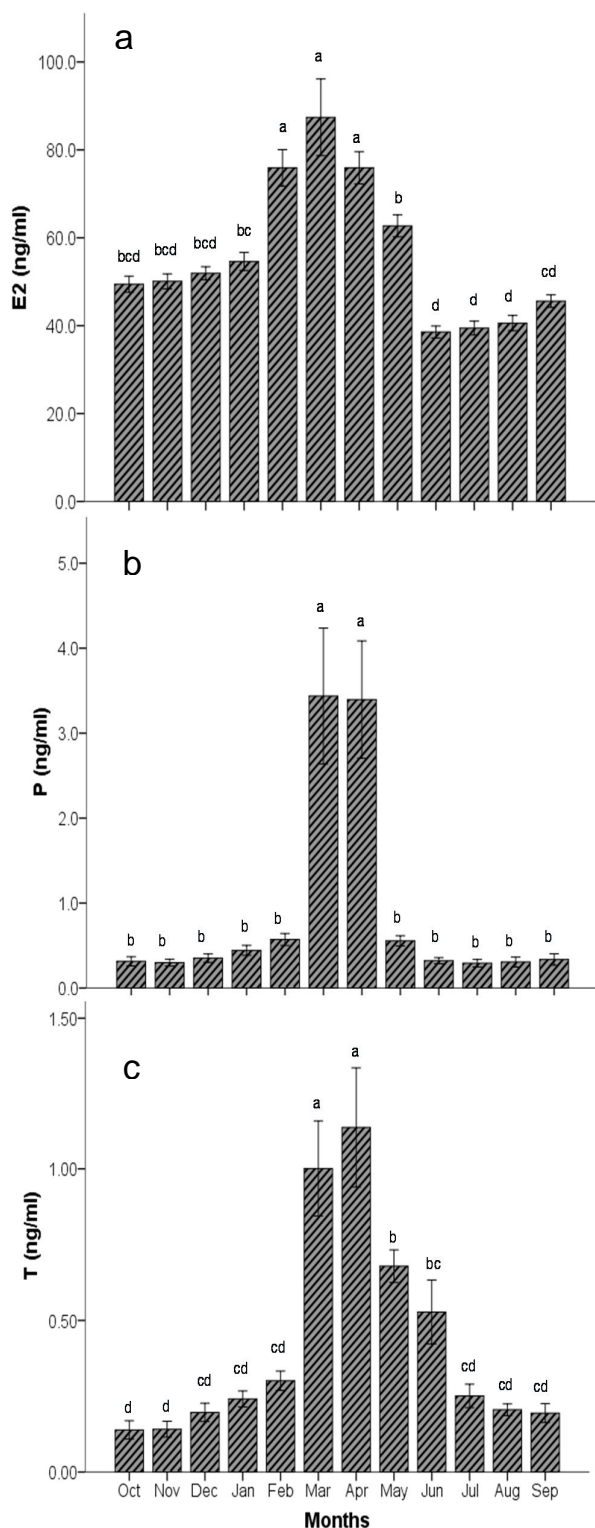


Figure 2. Effect of the temporal variations on sex steroid hormones in plasma content (ng/ml) from October to September; a) Estradiol, b) Progesterone and c) Testosterone. Mean annual plasma steroid hormone concentrations in *Rutilus frisii* Kutum from October to September. Different letters reveal significant differences (n= 106).

There was only slightly increase from January till February and then suddenly increased significantly during February and March. Post-hoc test, Duncan Multi comparison test results showed in table 1.

Table 1. Annual absolute numbers (Mean \pm S.E.M) of Gonadosomatic index (GSI) and plasma sex steroid hormones (ng/ml) of Estradiol, Progesterone and Testosterone in Caspian Sea Kutum (n=106).

Months	E2 (ng/ml)	T (ng/ml)	P (ng/ml)	GSI (%)
October	49.45 \pm 5.44	0.13 \pm 0.09	0.31 \pm 0.16	18.06 \pm 0.35
November	50.08 \pm 5.11	0.14 \pm 0.07	0.29 \pm 0.12	17.50 \pm 0.60
December	51.93 \pm 4.42	0.19 \pm 0.09	0.35 \pm 0.14	21.50 \pm 1.31
January	54.62 \pm 6.15	0.24 \pm 0.07	0.44 \pm 0.17	24.43 \pm 0.87
February	75.91 \pm 12.40	0.30 \pm 0.09	0.57 \pm 0.21	23.40 \pm 0.75
March	87.40 \pm 26.19	1.00 \pm 0.47	3.43 \pm 2.39	23.66 \pm 1.96
April	75.93 \pm 10.99	1.13 \pm 0.58	3.39 \pm 2.07	30.40 \pm 1.15
May	62.70 \pm 7.52	0.67 \pm 0.16	0.55 \pm 0.17	22.73 \pm 0.80
June	38.55 \pm 4.09	0.52 \pm 0.31	0.32 \pm 0.10	19.36 \pm 0.61
July	39.44 \pm 4.72	0.25 \pm 0.11	0.29 \pm 0.13	17.13 \pm 0.35
August	40.59 \pm 5.22	0.20 \pm 0.05	0.30 \pm 0.17	17.56 \pm 0.47
September	45.59 \pm 4.27	0.19 \pm 0.09	0.33 \pm 0.20	17.86 \pm 0.40

Moreover, Pearson linear correlation test revealed that there is a positive correlation between plasma estradiol levels (E2) and progesterone levels (P). Pearson linear relationship: 0.78. T= 13.11, df= 106, $P < 0.001$. (See figure 3a). Measurement of Testosterone content of plasma showed that there is a significant effect of temporal variations on T among months $F_{11, 96}:16.91$, $P < 0.001$. (See figure 2c). Plasma level of T increased to some extent from december till february and after that reached to the highest peak levels in march and april. Positive correlation was observed between level of estradiol (E2) and testosterone (T). Pearson linear relationship: 0.69. T=9.83, df=106, $P < 0.001$. (See figure 3b).

Maturity stages and gonad developments

According to the results of the present study, tissue sections in three areas, posterior, middle and end ovarian Kutum, there was no significant difference in the frequency of oocytes. Investigations with regard to the stages of growth and independence ovarian Kutum can be divided into six stages (see figure 4 and 6) including: first stage (stage nucleolus chromatin), the second stage (stage nucleolus side), the third stage (stage vesicles yolk), fourth step (step seeds yolk), the fifth stage (maturity stage) and stage six (oocytes stage). In all stages, follicular layer, cell wall, vacuoles, nuclear and nucleolus stage were then investigated.

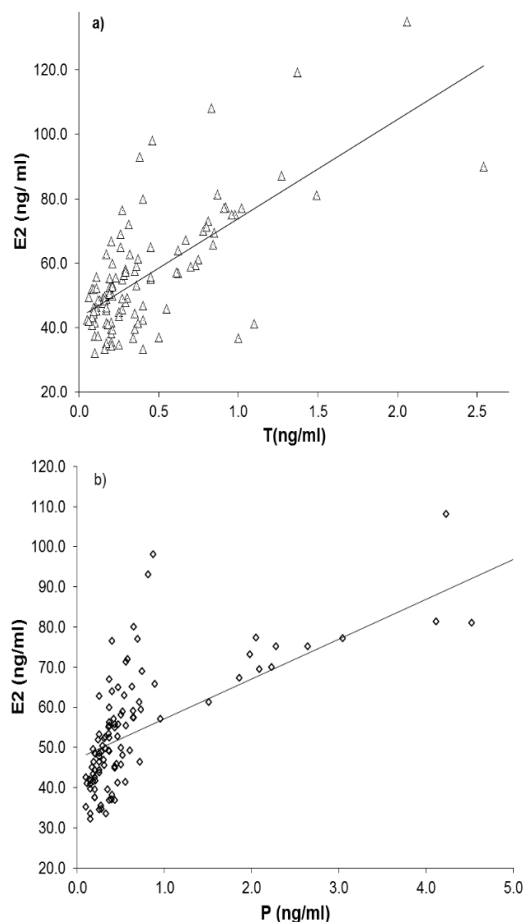


Figure 3. Positive correlation between plasma concentrations of E2 and P in Kutum and E2 and T in Kutum (n=106).

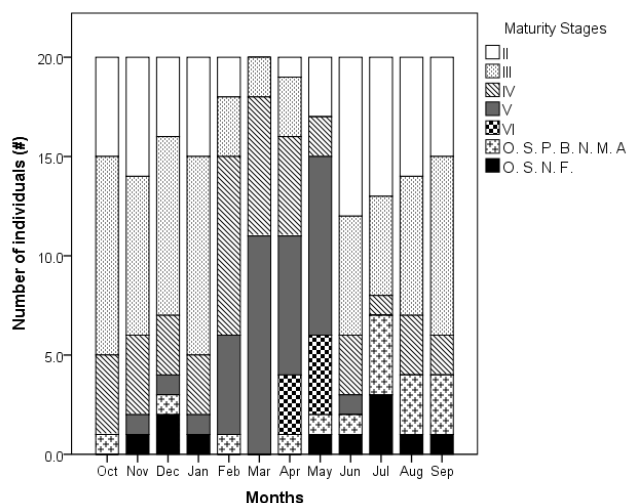


Figure 4. Stages of gonadogenesis and gonad development (II-VI) in Caspian Sea Kutum (n=20 per each month) from October to September.

Histological studies showed that Kutum had the same stages of oocyte development during migration season and this indicated synchronous oocyte development. Gonadosomatic index fluctuated among months and the highest level was observed in April; $F_{11,24}:56.20, P<0.001$. (See figure 5). Duncan multi comparisons test was applied for Post hoc analysis among months.

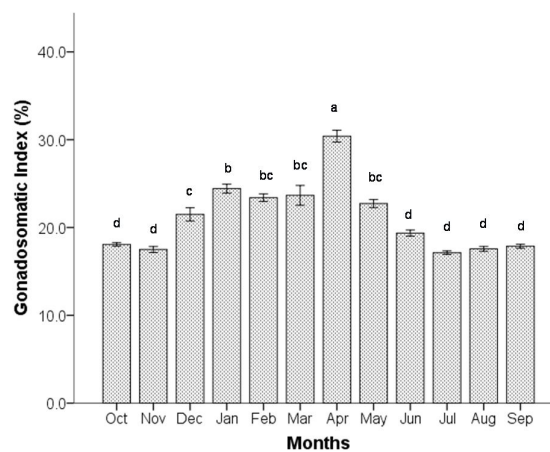


Figure 5. Annual fluctuations of the Gonadosomatic index (GSI) in Caspian Sea Kutum. (n=20 per each month) from October to September.

Discussion

The present study for the first time provides annual fluctuations of sex steroid hormones and gonad developments during reproduction cycle of the migratory Cyprinid Kutum. Our results revealed that there is a significant fluctuation of steroid hormones during annual reproduction cycle in Kutum. The highest peak level of steroid hormones were observed in spawning season; E2 (February – May), P (March – April) and T (march-April). During June- January which is the estivation stage, and also well known as foraging stage, when predominantly the ovary of the Kutum is small and present in stages II AND III, circulatory content of E2 and T were in low levels. Moreover, the level of steroid hormones significantly suppressed after the spawning period. From February concentration of E2 increased and subsequently in March concentration of T and P in plasma began to rise and reached immediately within the month at the maximum levels.

During this period in April the female individuals had reached to the maximum GSI when approximately oocyte maturation took place.

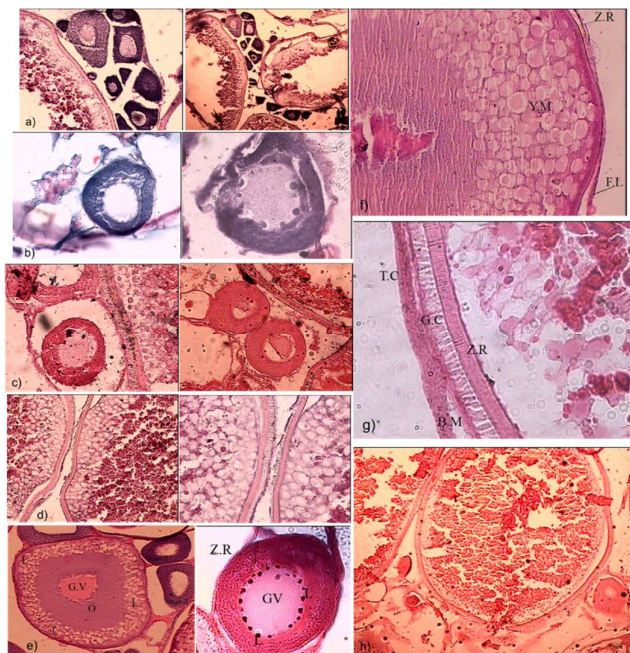


Figure 6. Histological observation of cyprinid fish, Kutum gonad development. a) Cross-section of ovary, stage I (of nucleus chromatin) 20x & 40x. b) Cross-section of ovary, stage II (nucleus and side) 20x & 40x. c) Cross-section of ovary, stage III (Vesicles yolk) 20x. d) Cross-section of ovary, stage IV (yolk grains) 20x. e) Light micrograph of an oocyte in the cortical alveoli phase from 5 μ m cross section in *Rutilus frisii kutum*. Lipid droplets (L) are present under the (ZR): zona radiata. GV: germinal vesicle. 70x. f) Light micrograph of an oocyte in the vitellogenic phase from 5 μ m cross section in *Rutilus frisii kutum*. YM: yolk material; ZR: zona radiata; FL: follicular layer. 480x. g) Light micrograph of follicular layers and oocyte in the maturation phase from 5 μ m cross section *R. frisii kutum*. ZR: zona radiata; GC: granulosa cell; TL: thecal layer; O: ooplasm. 525x. h) Oocytes in Stage V (mature) and i) Stage VI (eggs found or spawned)

The rapid increase in plasma steroids in Kutum was highly correlated with increases in the gonadosomatic index. After May, although the GSI continued to increase further and reached the highest values until April, plasma concentration of E2 exhibited a sharp decrease in April. There was a positive correlation between plasma level of E2 with P and T concentration. Gonad development had reached to the stage V as a final gonad maturation stage during February-May.

From February concentration of E2 increased and subsequently in March concentration of T and P in plasma began to rise and reached immediately within the month at the maximum levels. This higher level of E2 and T are coinciding with the vitellogenic procedures in ovary. The level of steroid hormones

diminished significantly after the spawning period. This part of the result is in line with earlier study on Indian major carp species *Labeo rohita* (Sen et al., 2002). Testosterone has been reported in the blood of a number of female Teleost such as cyprinid fish, Kutum (Heidari et al., 2010; Taghizadeh et al., 2013). Although testosterone and 11-ketotestosterone are male specific androgens, they are also present in blood plasma of female fish (Rinchart et al., 1993). As a precursor of E2 production, T is available in ovary for final female gonad aromatization. There is a relationship between testosterone and 17 β -estradiol in female fish. Testosterone leads to production of 17 β -estradiol, which in turn leads to vitellogenin production (Kagawa et al., 1983).

The GSI is an invaluable factor for monitoring the progression of gametogenesis in cyprinid fish, Kutum. High gonadosomatic indices recorded in April implied that gonads of Kutum were matured in this month and the females would likely spawn during these month in the wild (Heidari et al., 2010; Abdolhay et al., 2011; Taghizadeh et al., 2013). Moreover, earlier studies have shown that increments in ovarian gonadosomatic index and oocyte development are associated with changes in E2 levels in plasma (Lee and Yang 2002; Heidari et al., 2010; Adebisi et al., 2013; Taghizadeh et al., 2013). High gonadosomatic indices in the month April with high levels of E2 in March-April indicated that as maturation of oocytes progressed in the ovary, levels of E2 also increased in the plasma of Kutum. Thus, confirming the role of E2 in oocyte maturation and vitellogenesis (Scott et al., 1983; Rinchart and Kestemont 1996; Patiño et al., 2003; Adebisi et al., 2013).

In non-mammalian vertebrates, ovarian follicle cells produce two different steroid hormones, E2 and maturation inducing hormone (P), in response to pituitary gonadotropins, which play important roles in two phases of oogenesis, vitellogenesis and oocyte maturation, respectively. Estradiol-17 β (E2) promotes vitellogenesis in members of all non-mammalian vertebrates. In each of these groups, the time of vitellogenin production corresponds to the period of elevated E2 levels. Vitellogenin is the precursor molecule for egg yolk, which is of considerable importance as the source of metabolic energy for the developing embryo. In response to increased levels of plasma E2, the liver synthesizes and secretes vitellogenin, which is carried in the bloodstream to the oocytes (Nagahama 1994). The developing oocytes take up vitellogenin and convert it to egg yolk. On the other hand, a variety of progesterone have shown to be effective in the

initiation of meiotic maturation in fish and amphibian oocytes (Nagahama 1994; Kobayashi et al., 1996).

A sudden drop in the plasma E2 level in cyprinid fish, Kutum from vitellogenic stage to postvitellogenic stage may be explained in terms of swit Fostier off the arkobayomatase (CYP 19) activity as the oocytes progressed to maturation. Almost a similar pattern of fluctuations in E2 profile has been reported during the transition from vitellogenic to maturational stage in rainbow trout (Scott et al., 1983; Scott et al., 1984; Fostier and Jalabert 1986); amago salmon (Young et al., 1983); masu salmon (Yamauchi et al., 1984); coho salmon (Van Der Kraak and Donaldson, 1986); medaka (Sakai et al., 1987) and Indian major carp (Sen et al., 2002). In this context, however, it may be mentioned that in some other teleost such as a gudgeon, (Rinchard et al. 1993); Perciforms, (Prat et al., 1990) and cyprinid fish, goldfish, (Kagawa et al., 1983) there was no decrease of E2 level during oocyte maturation.

Our results showed that in another cyprinid fish species there is a drop of E2 levels during final period of oocyte maturation. Earlier study has shown that at high concentration, plasma level of T might also be involved in hepatic vitellogenin synthesis (Fostier and Jalabert 1986). Moreover, it is clear from our results that concentration of the sex steroids in plasma is dramatically different between spawning season migration and the rest of the reproductive cycle in Kutum. Several studies on structural changes in ovarian morphology of Teleost fishes have been done during oogenesis process (Guiguen et al., 1993; Prat et al., 1990; Scott et al., 1983; Sen et al., 2002; Shabana et al., 2012). Our histological measurements reinforce earlier study on gonad development of this cyprinid fish and indicated that the Kutum, *R. frisii kutum* exhibits synchronous group oocyte development (Heidari et al., 2010; Saeed et al., 2010).

The sexual mechanism of Teleost fish species is a complex aspect including gonad developments, maturation of oocytes, ovulation of mature eggs and subsequently spawning activities, which are regulated by the hypothalamus organ. This area of the brain produces an agent that releases gonadotropins hormones from the pituitary gland and regulates the target organs of the gonadotropins (Mojazi Amiri et al., 1996). Gonad structure of Kutum with the results obtained in this investigation were similar to other Teleost fish with the same spawning strategy (Lee and Yang, 2002; Manosroi et al., 2003). More studies are needed to investigate annual fluctuation of sex steroid hormones and their

relation with the gonad development. However, individual morphology and environmental factors seem to play an important role in this context.

In conclusion, , this study has investigated plasma sex steroid levels of the wild female Caspian Sea Kutum and the accompanying changes in gonadal development. from our results we conclude that in the wild cyprinid Kutum, sex steroids biosynthesis have significantly increased and reached to highest level during reproduction cycle at the onset of spawning migration in early spring. These hormonal changes are closely connected to the gonad development, maturity and subsequently gonadosomatic index. Based on information from plasma sex steroid hormonal profile, gonad histology and gonadosomatic index of the present study, it can be inferred that the annual reproductive cycle of Kutum did show a seasonal spawning pattern.

Interestingly our results for the first time revealed that this Cyprinid fish species show hugely high plasma E2 levels and high GSI even in non-reproductive fish individuals during non-reproductive seasons in June-September) with the basal levels of 40 ng/ml E2 (See figure 2a). And we can see a slightly increase in E2 levels during October –January with the basal levels of 55 ng/ml E2. We argue that this high levels of E2 may suggesting either non-migratory Kutum species populations inhabit in the Caspian Sea or Kutum species which migrate to adjacent wetlands not in spring but during autumn for spawning activities (Abdolhay et al., 2012; Rezvani Gilkolaei et al., 2011). The present study add the growing body of reproductive biology of one of the most important Cyprinid fish species, Kutum in Caspian Sea. Moreover, to achieve a successful artificial reproduction of Kutum it is important to explore further the impact of brood stock fish body size on temporal fluctuation of steroid hormones. More longtime period investigations are needed to establish a breeding protocol for Kutum population in captivity for restock management purposes.

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Putative RFLP Analysis Between HSVd-sycv and Closely Related Variants

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Abstract

Yellow corky vein is a prevalent disease among navel oranges in the Fars province of Iran. Previously we showed that a variant of Hop stunt viroid (HSVd-sycv) was associated with the disease. It was closely related to citrus variant of HSVd from Japan (HSVd-cit8) and with 93.7% homology with lime yellow corky vein variant of HSVd (HSVd-lycv). In this study, putative RFLP (Restriction Fragment Length Polymorphism) analysis using Vector NTI program (version 9.0.0) showed that some restriction enzymes could cut HSVd-sycv but not HSVd-lycv. On the other hand, *M.Ngo* BIX and *FauI* which could cut HSVd-lycv at positions of nt 53 and 97, respectively, could not cut HSVd-sycv. Likewise, some restriction enzymes could cut HSVd-cit8 but could not cut HSVd-sycv. Therefore these variants can be recognized by these restriction enzymes.

Keywords: HSVd-sycv, HSVd-lycv, Restriction Fragment Length Polymorphism analysis

Hop stunt viroid (HSVd), associated with serious disorders of economic importance, i.e. hop stunt, dapple fruit disease of plum and peach, apricot and jujube disease and citrus cachexia (Amari et al., 2000; Avina-Padilla et al., 2015; Gorsane et al., 2010). HSVd is one of the most widespread and economically important viroid of citrus plants (Su et al., 2015) and citrus strains of this viroid, have been implicated in yellow corky vein disease of Kagzi lime (*Citrus aurantifolia*), reported from India (Anirban Roy and Ramachandran, 2003).

The disease was characterized by yellow spots on leaf lamina, and rapid spread along the mid and lateral veins. The veins became rough on the lower surface of their leaves and developed corky tissues. A yield loss of 51.3–60.4% was reported from Assam (Azad, 1993). An isolate of HSVd (lime yellow corky vein variant; HSVd-lycv) was associated with the disease (Roy and Ramachandran, 2006). In recent years, a disease with specific signs of yellow corky vein has appeared in navel oranges in the Fars province of Iran. Previously we reported that a novel variant of HSVd (sweet orange yellow corky vein variant; HSVd-sycv) is constantly associated with the disease. The full sequence of the viroid was composed of 302 nucleotides. It was closely related to citrus variant of HSVd from Japan and with 93.7% homology with lime yellow corky vein variant

of HSVd-lycv. It was different in a single nucleotide from a noncachexia variant of HSVd from Japan (Bagherian and Izadpanah, 2010). In this research, putative Restriction Fragment Length Polymorphism (RFLP) analysis (Jamshidi et al., 2014) using Vector NTI program (version 9.0.0) showed that these related variants of HSVd could be recognized by some restriction enzymes.

Our previous nucleotide sequence data (Bagherian and Izadpanah, 2010) were used to predict mutual differential restriction enzymes between HSVd-sycv and closely related variants using Vector NTI program (version 9.0.0). *In silico* analysis by using Vector NTI program (version 9.0.0) proved that *Bbr*7I, *Bbs*I, *Bse*RI, *Cvi*AI, *Fat*I, *Hpy*CH4I, *M.Cvi*AI, *M.Cvi*QVII, *M.Cvi*SII and *M.Esa*BS1I restriction enzymes could cut HSVd-sycv but not HSVd-lycv. On the other hand, *M.Ngo*BIX and *Fau*I which could cut HSVd-lycv at positions of nt 53 and 97, respectively, could not cut HSVd-sycv (Figure 1). In the same manner, *M.Eco*RII, *M.Bst*NI, *M.Eco*KDcm and *Bst* NI could cut HSVd-cit8 but could not cut HSVd-sycv (Figure 2). Although viroid interactions are reported to alter plant reaction (Katsarou et al., 2015), none

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of the common citrus viroids except HSVd-sycv were detected constantly in yellow corky vein affected plants. Point mutation experiments and testing the variants on the same host must be carried out to verify the role of single nucleotide change in production of specific symptoms.

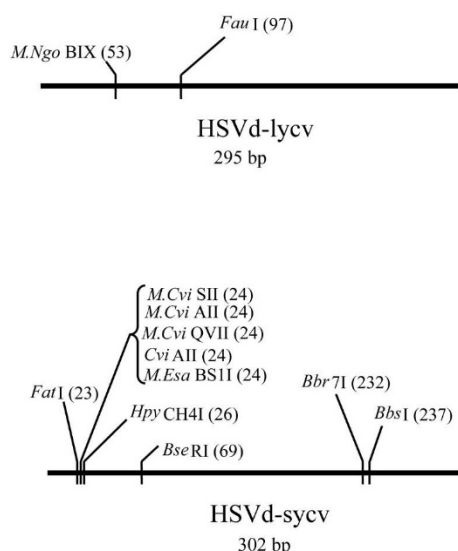


Figure 1. Putative restriction sites of HSVd-lycv vs HSVd-sycv using Vector NTI program (version 9.0.0) database.

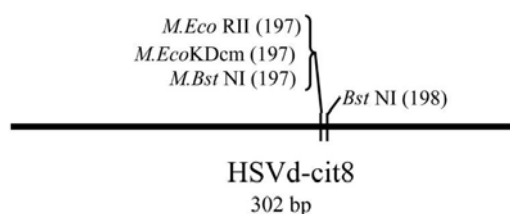


Figure 2. Restriction enzymes able to cut HSVd-cit8 but not HSVd-sycv according to *in silico* analysis using Vector NTI program (version 9.0.0) database.

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Targeted Cancer Therapy: A Hopeful Cure for Future

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Summary

Cancer is a multi-factorial disease that is spreading very fast in today's world. Informative activities and preventive efforts have been increased in recent years. Conventional therapies are used for patients to reach an acceptable level of success and patients can continue their lives but there are some side effects and most of the times the normal cells encounter damages by these therapies and also probability of recurrence always exist. So, the presence of a definitive therapy is needed to cure patients without these problems after therapy. Cancer cells need to use specific molecules in their cellular networks to continue their lives. Therefore, by elucidation of these molecules and targeting them purposefully a therapeutic strategy can be developed that is called targeted cancer therapy.

Keywords: Targeted cancer therapy, Multi-factorial disease, Specific molecules, Signaling network

Dear Editor...

There is no doubt that cancer is a global issue that is growing very fast in the world. This terrible disease causing millions of death every year, arises from fundamental perturbations in cellular signaling network that oblige cells to transform and finally establish a tumor. The cells can migrate to other sites of the body and constitute new encampments for attacking to other parts of the body that is called metastatic situation. In this stage it can be called cancer (Gupta and Massagué, 2006).

Cancer is a multi-factorial disease originating from several factors which also depends very much on life style. So, it is important to have a healthy life style to prevent this disease. (Belpomme et al., 2007; Jin et al., 2014). Nowadays, informative activities and preventive efforts have been increased but most of the time there is no way except therapy to rescue patients (World Health Organization, 2007).

Several molecules are changed during cancerous process and enormous studies have been performed to elucidate the crucial roles of these molecules in this process (Mishan et al., 2015).

Conventional therapies such as radiotherapy and chemotherapy have not offered successful and definitive results by producing side effects in cancer patients (Subramanian et al., 2015).

It is important to investigate for new therapies that could cure patients without side effects and recurrence.

In recent years, new treatments have been introduced for this purpose and among them, targeted cancer therapy have attracted many attractions. Three strategies have been introduced for targeted cancer therapy including antagonizing of specific molecules, targeted siRNA delivery and therapeutic monoclonal antibodies. By means of this therapy, molecules that are essential in cancerous process and also cancer cell viability are targeted and destroyed. Based on this theory, clinical trials have been performed with some promise but have not been used yet in clinic (Mishan et al., 2016).

So, it must be investigated how these methods could be used in clinic and how to establish targeted cancer therapy. By answering to these questions many hopes will be created in society towards absolute eradication of this terrible disease

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***Aegle marmelos* Leaf Extract is an Effective Herbal Remedy in Reducing Hyperglycemic Condition: A Pre-clinical Study**

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Abstract

Diabetes is one of the alarming health problem in Pakistan. According to international diabetes federation (IDF), about 7 million Pakistanis are victims of diabetes accounting a total of 3% population. Most of Pakistanis still prefer to use herbal medicines for almost all of their health issues. *Aegle marmelos* (commonly known as the bael tree) is highly reputed ayurvedic medicinal tree as it has been used in traditional medicine for centuries. In this pre-clinical work, 32 mice were induced for diabetes via injecting alloxan intraperitoneally at a dose of 100 mg/kg body weight and mice having 104-170 mg/dl sugar level were considered diabetic. All mice were divided into two groups, each group with three sub-groups containing normal, control and diabetic mice. One group was given normal feed whereas other group was given a food containing *Aegle marmelos* leaf extract at 300 mg/kg body weight as an effective dose against alloxan induced hyperglycemia. The experiments lasted for 60 days and blood samples (0.5 ml to 1.0 ml) were collected from coccygeal vein of rats on 1st day, 30th day and 60th day of the experiment in heparinized tubes and blood glucose level was measured using spectrophotometer by enzymatic kit at a wavelength of 540nm. It was observed that the *Aegle marmelos* leaf extract is an effective herbal remedy in reducing and maintaining the glucose level in normal and hyperglycemic mice.

Keywords: Diabetes mellitus, *Aegle marmelos* leaf, Hyperglycemia, Pre-clinical study

Introduction

Diabetes mellitus (DM) is a major endocrine disorder of carbohydrate disturbed metabolism and growing health problem in the world. It has been suggested that formation of free radicals is involved in the pathogenesis of diabetes and in the development of diabetic complications because a prolonged exposure of the antioxidant defense system to hyperglycemic condition (Sander *et al* 2001). It is also a syndrome problem resulting from variable interactions of hereditary and environmental factors and characterized by the depleted insulin secretion, hyperglycemia and altered metabolism of lipids, carbohydrates and proteins, in addition to damaged β -cells of pancreas and an increased risk of complications of vascular diseases. Hence, it is characterized by high level of glucose in the blood resulting from defects in insulin production (insulin deficiency), insulin action (insulin resistance) or both (Davis and Granner, 1996, Ruth *et al.*, 1999, Robert *et al.*, 1999). Insulin (Latin insula, "island") is a polypeptide hormone primarily playing a pivotal role in the regulation of

carbohydrate metabolism as well in metabolism of fats and proteins. Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species causing an insulin-dependent diabetes mellitus called alloxan diabetes in these animals that is similar to type 1 diabetes in humans (Berg *et al* 2001, Fuller *et al.*, 1990, Ruddon and Gilman, 2000).

According to the latest statistics given by jointly Pakistan diabetes federation (PDF) and international diabetes federation (IDF), Pakistan has 7 million diagnosed diabetes patients on an average of 3% of national population which is ranked 14 in case of diabetes prevalence in the world (<http://www.idf.org/membership/mena/pakistan>) as shown in figure 1. Presently control of diabetes mellitus relies on the injection of IV insulin and the usage of many chemicals and plant extracts in subcontinent. A number of plants have been studied for this purposes and their extracts were prepared and are available in market as remedy for diabetes

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(Vats *et al.*, 2002). Some medicinal plants have been found playing extra pancreatic effect and acting directly on liver, muscle tissues altering the activities of the regulatory enzymes of glycolysis, gluconeogenesis and other pathways. Since the plant products have fewer side effects, so these have the potential as good hypoglycemic drugs and can provide us clues for the development of new and better oral drugs (Shukia *et al.*, 2000, Mukherjee *et al.*, 2006).

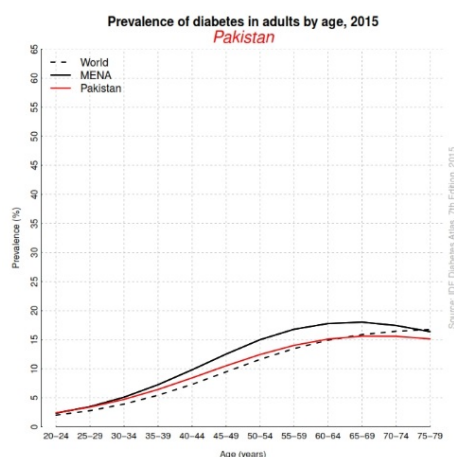


Figure 1. Prevalence of diabetes and its comparison with world and Middle East region (MENA). (<http://www.idf.org/membership/mena/pakistan>)

The medicinal values of plants have been tested by trial and error method for a long time by different workers. Even today great opportunities are still open for scientific investigations of herbal medicines for the cure of diabetes and its complications. *Aegle marmelos* (AM) commonly known as Bael is a spiny tree belonging to class Rutaceae. It is an indigenous tree found in India, Myanmar, Pakistan and Bangladesh. The leaves, roots, bark, seeds and fruits are edible and have medicinal values as described in the Ayurveda (Khair, 2004) and can act as a hypoglycemic agent (Alam *et al.*, 1990, Grover *et al.*, 2002, Mukherjee *et al.*, 2006, Kamalakkannan and prince, 2003, Kesari *et al.*, 2006).

Preliminary reports indicate blood glucose lowering agents in green leaves of *Aegle Marmelos* plants. However, limited scientific evidence exists to validate these claims since there are only few available reports on the exact mode of action of these extracts and pharmacological actions of this plant (Ponnachan *et al.*, 1993. Das *et al.* 1996; Schdewa *et al.* 2001; Nema *et al.* (1991) Riyanto *et al.* (2001) Upadhya *et al.* 2004, Kesari *et al.* 2006). This work was aimed to study the glucose lowering effect of AM leaves in different groups of diabetic animals.

Materials and Methods

The experimental work was conducted in animal house of the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan.

Aqueous leaves extract of *Aegles Marmelos* (Bael)

Plant material and Preparation of Extract

Aegle marmelos leaves were collected from the Botanical garden of Punjab University and thoroughly washed with water and dried in shade. Then these leaves were grinded to make powder and were kept in airtight containers for the experimental purpose. About 500 g of the shade dried powdered leaves were ground into fine powder and boiled in 5 liters of distilled water for 7-8 hours and stirred occasionally. After this, the mixture was filtered using Whatmann No. 1 filter paper and 50 g of the paste was obtained. Then this paste was dissolved in 0.5 ml water just prior to the administration.

Induction of diabetes in mice

Alloxan induced hyperglycemia has been described as a useful experimental model to evaluate the activity of hypoglycemic agents (Junod *et al.* 1996). Diabetes was induced by a single intraperitoneal injection of alloxan prepared in 0.1 mol/L citrate buffers at a dose of 100 mg/kg body weight. Diabetes was confirmed in the alloxan treated rats by measuring the fasting blood glucose concentration 48 h of post injection as described by other researchers (Karunanayake *et al.* 1984 and Grover *et al.* 2002).

Enzymatic Kits

Commercial kits of the company Randox, UK were used to determine serum glucose in mice by spectrophotometer.

Animals

Total 32 mice of both sexes weighing between 35-36 g were selected for the experiment. These animals were housed in steel cages under controlled laboratory conditions. The mice were fed standard diet with free access to fresh water (Reeves *et al.*, 1993)

Experimental Design

32 rats were divided into four groups (A, B, C and D) with eight mice in each group. Each group was having both normal and diabetic mice as described in table 1.

Table 1. Animal grouping, their diet and treatment research plan

Groups		Animal Conditions	Treatment
1	A	Normal (control)	Normal feed (3% b.w.)
2	B	Normal + (<i>Aegle Marmelos</i>)	Normal feed + aqueous Leaves extract of <i>Aegle Marmelos</i> (300 mg/kg).
3	C	Diabetic (control)	Normal feed (3 % b.w.)
4	D	Diabetic + (<i>Aegle Marmelos</i>)	Normal feed (3% b.w.) + aqueous Leaves extract of <i>Aegle Marmelos</i> (300 mg/kg).

Blood Collection

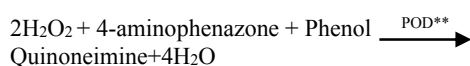
1 ml blood was collected after 12 h of fasting from coccygeal vein of mice at 1st day, 21st day and 42nd day of the experiment from.

Blood Analysis

Collected blood was allowed to clot and then centrifuged at 3000 r.p.m. for 10 minutes and serum was separated. The specific enzymatic kits were used to assess serum glucose levels of mice using spectrophotometer.

Estimation of Serum Glucose**Principle**

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a Reddish-pink quinoneimine dye as an indicator.

Reaction Principle

*GOD Glucose oxidase

**POD Peroxidase

Sampling

Glucose is stable for 24 hours at +2 to +8°C if the serum was prepared within 30 min after collection.

Reagents and Parameters**Reagents**

1. Enzyme Reagent
2. Standard (100 mg/dl)

Standard	Blank	Standard	Tests
(100 mg/dl)	----	10 µl	----
Tests	----	----	10 µl
Enzyme reagent	1 ml	1 ml	1 ml

Parameters of Study

Wavelength λ: 500 nm, Hg 546 nm

Cuvette: 2 cm path length

Temperature: 15-25°C or 37°C

Measurement: against reagent blank

10 test tubes were taken. Two tubes out of 10 test tubes were labeled as blank and standard. Remaining 8 tubes were labeled as 1, 2, 3....8 for each sample of mice serum from each group. 1ml reagent was taken in all the tubes by pipette. 10 µl of standard solution from the kit was added to the tube labeled as standard and 10 µl of each serum sample was taken in tubes labeled as 1, 2, 3,....8.

All the tubes were shaken well and then incubated at 37°C for 10 minutes. After incubation, the absorbance of standard (AbsStd) and the sample (AbsS) was measured at wavelength of 546 nm against the blank (AbsSRB).

Normal values

Serum, plasma (fasting): 75-115 mg/dl

Linearity

This method is linear upto 400 mg/dl. Samples having higher glucose concentration were diluted at 1:2 with saline solution and the measured calculation was multiplied by 2.

Calculations

The concentration of glucose in serum was calculated by the following formula;

$$\text{Glucose conc. (mg/dl)} = (\Delta \text{ test} / \Delta \text{ standard}) \times 100$$

Statistical Analysis

The data thus obtained was subjected to statistical analysis represented by mean ± S.D for 8 mice in each group of experiments. Comparison among the different groups was determined by ANOVA test

and differences were considered significance when $p < 0.05$. The level of significance was set at 5% according to the method of Steel and Torri, 1982.

Results

The present work was carried out to investigate the effect of feeding *Aegle marmelos* leaves on serum glucose level in diabetic mice.

Serum Glucose level (mg/dl)

The change in blood glucose level of mice blood serum as a function of the *Aegle marmelos* leaves extract for a feeding period of 8 weeks is presented in figure 2.

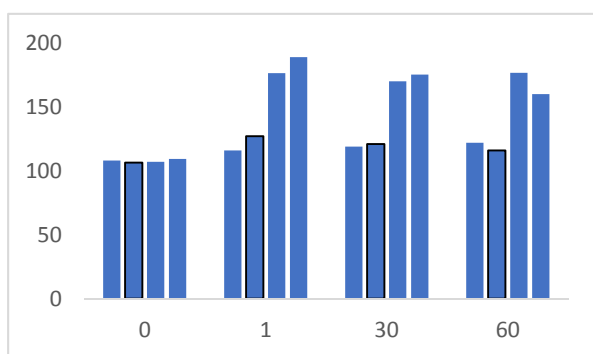


Figure 2. Change in blood glucose level of mice blood serum

In control group A, the mean glucose level was 108.2 ± 7.28 before alloxan injection, which increased to 116.1 ± 8.53 at 1st day after treatment and then further raised to 119 ± 8.26 at 30th day and 122 ± 8.42 at 60th day.

In normal group B, the mean glucose level was 106.5 ± 9.81 before alloxan injection, which increased to 127.14 ± 8.23 at 1st day after alloxan injection and then further raised to 121 ± 8.26 at 30th day and 116.1 ± 8.53 at 60th day.

In diabetic control group C, the mean glucose level was 107.2 ± 6.99 before alloxan injection, which increased to 176.5 ± 3.64 at 1st day and then further raised to 170.2 ± 6.10 at 30th day and 176.8 ± 10.97 at 60th day.

In diabetic group D, the mean glucose level was 109.4 ± 6.55 before alloxan, which increased to 189.1 ± 8.02 at 1st day and then decreased to 175.4 ± 12.62 at 30th day, and further decreased to 160.1 ± 7.33 at 60th day.

According to the analysis of variance, at before given alloxan, Non-significant difference was observed in Group A (control + normal feed) as $P > 0.05$, while at day 1, 30 and 60 of the experiment there was significance difference among the mean value of glucose level in the groups i.e. Group B (Normal + *Aegle marmelos* leaf extract), Group C

(Diabetic + normal feed) and Group D (Diabetic + *Aegle marmelos* leaf extract) as $P < 0.05$ (Figure 3).

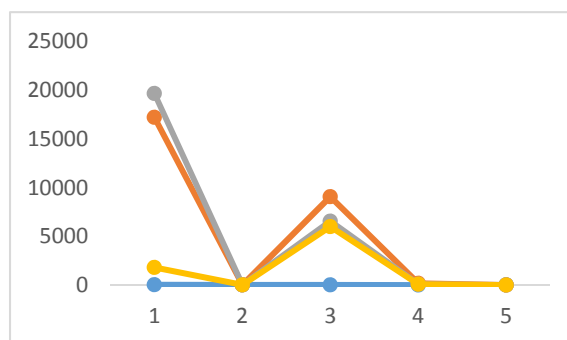


Figure 3. Analysis of Variance of Glucose level

At day zero before alloxan injection, non-significant difference was observed in groups i.e. Group A (control + normal feed, group), Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML), Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P > 0.05$ (Table S1).

At 1st day of experiment, significant difference was observed in groups i.e. Group A (control + normal feed), Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML), Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P < 0.05$. At 30th day of experiment, non-significant difference was observed in Group A (control + normal Feed), Group C (diabetic + normal feed) with Group B (normal + aq. leaves extract of AML) and Group D (diabetic + aq. leaves extract of AML) respectively as $P > 0.05$, while significant difference was observed in Group A (control + normal feed) with groups i.e. Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) and Group B (normal + aq. leaves extract of AML) with groups i.e. Group B (diabetic + normal Feed) and Group C (diabetic + aq. leaves extract of AML) as $P < 0.05$.

At 60th day of experiment, Non-significant difference was observed in Group A (control + normal feed) with Group B (normal + aq. leaves extract of AML) as $P > 0.05$, While significant difference was observed in Group A (control + normal feed) with Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) and also significance difference observed in groups i.e. Group B (normal + aq. leaves extract of AML) and Group C (diabetic + normal feed) with groups i.e. Group C (diabetic + normal feed) and Group D (diabetic + aq. leaves extract of AML) as $P < 0.05$.

Discussion

Diabetes arises from destruction of the β -islet cells of the pancreas, due to degradation or reduction of insulin secretion (Ramkumar *et al* 2004). The elevation in plasma insulin in the *Aegle marmelos* leaf extract treated Alloxan-diabetic mice could be due to the insulin tropic substances present in the fractions, which induce the intact functional β -cells of the Langerhans islet to produce insulin, or the protection of the functional β -cells from further deterioration so that they remain active and produce insulin (Bell and Hye, 1983).

Diabetic mice induced by alloxan show an increased sensitivity to oxygen free radicals and hydrogen peroxide, the breakdown products of the liver, which impose oxidative stress in diabetes and would damage inner endothelial tissue; this would eventually be directly responsible for high blood glucose (Reddi and Bollineni, 2001). Alloxan and STZ are widely used to induce experimental diabetes in animals. The mechanism of their action in β -cells of pancreas has been intensively investigated and now is quite well understood. Experimental evidences suggest that the free radicals and reactive oxygen species are involved in high number of diseases. Free radicals (Ca^{2+} , Fe^{2+} , Fe^{3+} , H_2O_2 , Hydroxyl Oxide and Nitric Oxide inhibit Aconitase activity and participate in DNA damage of β -cell (Szkudelski, 2001).

The present research work was aimed to study the effect of *Aegle marmelos* leaf extract on serum glucose level in mice. The outcomes of present study showed that *Aegle marmelos* leaf lowers the glucose level in hyperglycemic mice. The experimentally induced diabetes significantly ($P < 0.05$) increased the fasting blood glucose level of the control level. However, the treatment of Alloxan-induced diabetic mice with the AML reduced their blood glucose levels, in comparison to the diabetic group.

This study showed that AML-extract supplementation improved glucose tolerance in diabetic mice. It seems that the hypoglycemic effect of AML is due to the increased level of serum insulin and the enhancement of peripheral metabolism of glucose (Skim *et al* 1999). The present experiment

reports the anti-diabetogenic and hypoglycemic effects of aqueous extract of *Aegle marmelos* leaves on alloxan or streptozotocin-induced diabetic mice. Loss of body weight had also been observed very distinctly in alloxan induced diabetic mice of the present study. After treatment with *Aegle marmelos* plant leaf extract, the mice regained their weight, which is close to the control (non-diabetic mice)

level which was also reported previously in 2004 (Satishsekar and Subramanian, 2005).

This was also confirmed by the alteration in the fasting blood glucose levels in diabetic mice followed by its regeneration after the plant extract treatment, then there is no significance alteration in fasting blood glucose level in the control mice. Further the same extract causes the significance reduction of sugar within 2 hours and this fact strengthens the antidiabetogenic potentiality of this plant extract (Karunanayake *et al* 1984, Grover *et al* 2002). The aqueous extract of *Aegle marmelos* contains some biomolecules that sensitize the insulin receptors to insulin or stimulate the β -cells of islets of Langerhans to release insulin which may finally lead to improvement of carbohydrate metabolizing enzymes towards the re-establishment of normal blood glucose level (Gupta *et al* 2005)

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Table s1. Multiple Comparisons of glucose level among different groups

Dependent Variables	Groups		Mean Difference	Std. Error	Sig.
Before Alloxan	Group A (Control) + Normal Feed	(Group B) (Normal) + aq. Leaves extract of AML	1.7143	4.22054	.688**
		(Group C) Diabetic + Normal Feed	1.0000	4.22054	.815**
		(Group D) Diabetic + aq. Leaves extract of AML	-1.1429	4.22054	.789**
	Group B (Normal) + aq. Leaves extract of AML	(Group C) Diabetic + Normal Feed	-.7143	4.22054	.867**
		(Group D) Diabetic + aq. Leaves extract of AML	-2.8571	4.22054	.505**
	Group C Diabetic + Normal Feed	(Group D) Diabetic + aq. Leaves extract of AML	-2.1429	4.22054	.616**
Day 1st	Group A (Control) + Normal Feed	(Group B) (Normal) + aq. Leaves extract of AML	-11.0000	3.94951	.010*
		(Group C) Diabetic + Normal Feed	-60.4286	3.94951	.000*
		(Group D) Diabetic + aq. Leaves extract of AML	-73.0000	3.94951	.000*
	Group B (Normal) + aq. Leaves extract of AML	(Group C) Diabetic + Normal Feed	-49.4286	3.94951	.000*
		(Group D) Diabetic + aq. Leaves extract of AML	-62.0000	3.94951	.000*
	Group C Diabetic + Normal Feed	(Group D) Diabetic + aq. Leaves extract of AML	12.5714	3.94951	.004*
Day 30th	Group A (Control) Normal Feed	(Group B) (Normal) + aq. Leaves extract of AML	-2.0000	4.87845	.685**
		(Group C) Diabetic + Normal Feed	-51.2857	4.87845	.000*

		(Group D) Diabetic + aq. Leaves extract of AML	-56.4286	4.87845	.000*
	Group B (Normal) + aq. Leaves extract of AML	(Group C) Diabetic + Normal Feed	-49.2857	4.87845	.000*
		(Group D) Diabetic + aq. Leaves extract of AML	-54.4286	4.87845	.000*
	Group C Diabetic + Normal Feed	(Group D) Diabetic + aq. Leaves extract of AML	-5.1429	4.87845	.302**
Day 60th	Group A (Control) Normal Feed	(Group B) (Normal) + aq. Leaves extract of AML	6.5714	4.76595	.181**
		(Group C) Diabetic + Normal Feed	-54.1429	4.76595	.000*
		(Group D) Diabetic + aq. Leaves extract of AML	-37.4286	4.76595	.000*
	Group B (Normal) + aq. Leaves extract of AML	(Group C) Diabetic + Normal Feed	-60.7143	4.76595	.000*
		(Group D) Diabetic + aq. Leaves extract of AML	44.0000	4.76595	.000*
	Group C Diabetic + Normal Feed	(Group D) Diabetic + aq. Leaves extract of AML	16.7143	4.76595	.002*

** = Non-significant value as $P > 0.05$

* = Significant value as $P < 0.05$

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