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Analysis of synonymous codon usage bias and nucleotide and amino acid composition in 13 species of Flaviviridae

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Abstract

The family, Flaviviridae includes viruses which cause several diseases including Dengue fever, Japanese encephalitis, Murray Valley encephalitis, Tick-borne encephalitis, West Nile encephalitis, Yellow fever and Hepatitis C virus infection. Members of this family have monopartite, linear, single-stranded RNA genomes of positive polarity with 9.6-12.3 kb in length. Here, we analyzed the codon usage of 13 species of this family by using gene infinity package. Base analysis was performed by CAIcal server and amino acid composition was calculated by PseAAC web-server. The results showed that the highest number of A, G and C bases were seen in the RNA genome of *Dengue* virus 2, *Tick borne encephalitis* virus and *Hepatitis C* virus, respectively. Although the number of U bases used in RNA genomes was very close, the highest U nucleotide amount was 23.77% in *Wesselsbron* virus. The lowest number of C, G, U and A bases was seen in *Bovine viral diarrhea* virus, *Dengue* virus 2, *Tick borne encephalitis* virus and *Hepatitis C* virus, respectively. In this study, it was found that the complete genome of *classical swine fever* virus has a lower GC content and genome of *Tick borne encephalitis* virus, *Hepatitis C* virus and *Powassan* virus have the highest GC content among other examined species. We also classified the amino acids as rare (Phenylalanine, Cysteine, Histidine, Methionine, Asparagine, Glutamine, Tryptophan and Tyrosine), frequent (Alanine, Glutamic acid, Glycine, Leucine, Valine and Threonine), and intermediate (all others). The highest numbers of preferred codons exist in *Wesselsbron* virus and the lowest in *West Nile* virus.

***Keywords*:** Flaviviridae, codon usage bias, amino acid composition, nucleotide composition

Introduction

Each amino acid is coded either by one or more codon(s). Therefore, genes and species may utilize different sets of codons (Vicario et al., 2007). It has been demonstrated that codon usage pattern is nonrandom and species-specific, and the inter-genomic variation of the codon usage pattern is a common phenomenon (Grantham et al., 1981). For example, a range of minimal to extreme codon bias is present in unicellular organisms and in Drosophila species (Sharp and Li, 1987; Powell and Moriyama, 1997). In addition, it is well identified that different genes have special codon usage patterns in a same organism (Zhuo-Cheng et al., 2003). Some factors such as translational selection (Bennetzen et al. 1982), mutation (Sueoka et al., 2000), compositional constraints (Hou et al., 2002), physical location of the gene on chromosome (Kerr et al., 1997), replication-translational selection (Naya et al., 2001), hydrophobicity of each gene (Romero et al., 2000), gene length (Sau et al., 2005), and CpG island (Shackelton et al., 2006) were found to influence codon usage in animal viruses and phages. Mutational bias was found as the main determinant factor (XU et al., 2008).

Flaviviridae are mainly classified into three genera: Flavivirus, Hepacivirus and Pestivirus. Members of this family have monopartite, linear, single-stranded RNA genomes of positive polarity with 9.6-12.3 kb in length (Regenmortel et al., 2000). Major diseases caused by the *Flavivirus* and *Hepacivirus* genera include: Dengue fever, Japanese encephalitis, Murray Valley encephalitis, Tick-borne encephalitis, West Nile encephalitis, Yellow fever and Hepatitis C Virus Infection while *Pestiviruses* are usually pathogens of non-human mammals (Gould et al., 2001). Analysis of codon usage patterns of Flaviviridae would provide a foundation for understanding the related mechanism for biased usage of synonymous codons, the evolution and pathogenesis of Flaviviridae. It would be helpful for choosing appropriate host expression systems for an

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optimized expression of target genes. Here, using the codon usage database, the codon usage and base composition of thirteen species of Flaviviridae were analyzed, and the patterns of preferred codons for each individual amino acid in each species were identified.

Materials and Methods

Genomic sequence retrieval

Complete (or nearly complete) genomic RNA sequences for 13 Flaviviridae species and the amino acids of polyprotein sequences were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/). The accession numbers of these proteins are AY835999 (*Dengue* virus 1), GQ868604 (*Dengue* virus 2), DQ863638 (*Dengue* virus 3), AF045551 (*Japanese encephalitis* virus), NC_000943 (*Murray Valley encephalitis* virus), GQ228395 (*Tick-borne encephalitis* virus), AY640589 (*Yellow fever* virus), FJ407092 (*Hepatitis C* virus), AY842931 (*West Nile* virus), AF526381 (*Bovine viral diarrhea* virus), NC_012735 (*Wesselsbron* virus), GQ923951 (*Classical swine fever* virus) and EU770575 (*Powassan* virus). Polyprotein sequences were obtained from NCBI, including those of *Japanese encephalitis* virus (AAA21436), *Murray Valley encephalitis* virus (NP_051124), *West Nile* virus (AAV54504), *Tick-borne* encephalitis virus (NP_043135), *Powassan* virus (NP_620099), *Yellow fever* virus (AAT58050), *Wesselsbron* virus (YP_002922020), *Hepatitis C* virus (BAB32877), *Classical swine fever* virus (ACL80334), *Bovine viral diarrhea* virus (NP_040937), *Dengue virus* 1 (AAV97946), *Dengue virus* 2 (ACW82981) and *Dengue virus* 3 (ACW83004).

Sequence analysis

The nucleotide compositions were determined by using CAIcal server. This server is available at http://genomes.urv.es/CAIcal. Codon usage data and effective number of codons (ENC) was analyzed by gene infinity package (http://www.geneinfinity.org). PseAAC web-sever was used to demonstrate amino acid composition of proteins (Shen and Chou, 2008).

Results

Base composition analysis

In order to examine the base composition variation among different complete RNA genome of several species of 13 Flaviviridae genera, their nucleotide composition was calculated.

As the results in table 1 indicate, strong nucleotide biases were observed in all of the examined genomes. The highest base percentage was 33.38 % A in *Dengue* virus 2, 31.57% G in *Tick borne encephalitis* virus and 28.56% C in *Hepatitis C* virus. Although the number of U base used in RNA genomes was very close, the highest U nucleotide amount was 23.77% in *Wesselsbron* virus. The lowest number of bases was 19.85% C in *Bovine viral diarrhea* virus 1; 25.17% G in Dengue virus 2; 20.70 U in *Tick borne encephalitis* virus, and 21.14 % A in *Hepatitis C* virus. The Adenine percentage was clearly the most frequent and nucleotide among these Flaviviridae members (ranging from 21.14 to 33.38%).

Table 1. Nucleotide composition of Flaviviridae.

Virus	Nucleotide				
	G%	% A	U%	C%	Length (bp)
Dengue virus 1	25.72	32.14	21.38	20.74	10727
Dengue virus 2	25.17	33.38	21.23	20.33	10679
Dengue virus3	25.98	32.04	21.20	20.77	10707
Japanese encephalitis virus	28.44	27.53	21.00	23.01	10963
Murray Valley encephalitis virus	27.51	28.89	22.40	21.20	11014
West Nile virus	28.76	27.34	21.49	22.40	11029
Tick borne encephalitis virus	31.57	24.95	20.70	22.76	11096
Powassan virus	31.19	25.20	21.49	22.12	10839
Yellow fever virus	28.33	27.40	22.92	21.35	10862
Wesselsbron virus	26.62	28.58	23.77	21.03	10812
Hepatitis C virus	27.75	21.14	22.55	28.56	9432
Classical swine fever virus	26.41	30.92	21.65	21.00	12296
Bovine viral diarrhea virus 1	26.31	31.89	21.79	19.85	12220

Table 2 shows that the complete genome of *Classical swine fever virus* has a lower GC content (42.9%) and genomes of *Tick borne encephalitis* virus, *Hepatitis C* virus and *Powassan* virus have a higher GC content than other species (While the corresponding percentages were 56.52, 56.31 and 53.32). No clear difference in GC content was seen among other genomes. On the other hand, differences in GC content at different synonymous positions of codons were obvious.

For example, GC content at the synonymous first position of codons for the *Classical swine fever* virus and *Yellow fever* virus are 37.25 and 36.02 %, respectively, while these values for *Tick borne encephalitis*, *Bovine viral diarrhea* and *Japanese encephalitis* viruses are 28.27, 28.33 and 29.00 %, respectively. The GC content at the synonymous second position of codons is calculated 28.72 % for *Powassan* virus but for the *Tick borne encephalitis* virus is 36.35 %. GC content at the synonymous third position of codons is computed 28.07 and 28.65 % for *Classical swine fever* virus and *Yellow fever* virus, respectively, while for the *West Nile* virus and *Dengue* virus 2 are 36.32 and 36.23 %. The most variable genome in GC content at the different synonymous positions of codons is *Classical swine fever* virus (ranging from 28.07 to 37.25%) but least variation was seen in RNA genome of *Wesselsbron* virus.

Amino acid composition

In this study, the frequency of the 20 amino acids in the polyproteins of different species of Flaviviridae was calculated (table 3). The amino acids can be classified as rare (Phenylalanine, Cysteine, Histidine, Methionine, Asparagine, Glutamine, Tryptophan and Tyrosine), frequent (Alanine, Glutamic acid, Glycine, Leucine, Valine and Threonine), and intermediary (all others).

GCN codon encodes Alanine and CCN codon encodes Proline residues. Interestingly, *Hepatitis C* virus with the highest C and G content has the largest number of Alanine and Proline residues. Lysine amino acid is encoded by two AAG and AAA codons. The Largest number of this amino acid was present in *Pestivirus* that had high A and low C content.

Codon usage patterns

Commonly, codons which were used more than twice as frequently as host consensus codons are regarded as preferred codons of heterologous genes (Lu et al., 2005). In this work, compared with *Homo sapiens* (selected host for these viruses), several preferred codons were found in each species (table 4). In *Dengue* virus 1, 3 and *Hepatitis C* virus five preferred codons and in *Bovine viral diarrhea* virus and *Dengue* virus 2 three preferred codons were demonstrated. The highest numbers of preferred codons exist in *Wesselsbron* virus and the lowest in *West Nile* virus. Six and seven preferred codons were found in *Yellow fever* virus and *Classical swine fever* virus while only two preferred codons were found in *Murray Valley encephalitis* and *Powassan* viruses. Four preferred codons were also characterized for *Japanese encephalitis virus*. AGA coding for Arg was preferred in most of the species. The ATA coding for Ile, CAC for His and TTG for Leu are preferred only in *Bovine viral diarrhea* virus, *Hepatitis C* virus and *Murray Valley encephalitis virus*. For Leu, RNA genomes of various species did not have any preferred codons while for Gly, Ser and Arg, they used two preferred codons in some species. The preferentially used codons were A-ended, T-ended, and G-ended. Flaviviridae can be classified in five species groups based on their genome signature (U-rich, G-rich, A-rich, C-rich, or relatively unbiased). Results are summarized in table 5.

Table 2. GC, A, T, G and C contents at different codon positions of complete genome of Flaviviridae

N%	*Dengue virus 1*	*Dengue virus 2*	*Dengue virus 3*	*Japanese encephalitis virus*	*Murray Valley encephalitis virus*	*West Nile virus*	*Tick borne encephalitis virus*	*Powassan virus*	*Yellow fever virus*	*Wesselsbron virus*	*Hepatitis C virus*	*Classical swine fever virus*	*Bovine viral diarrhea virus 1*
A1	35.60	28.77	34.12	27.37	27.32	30.66	26.04	27.01	23.59	29.28	18.06	27.72	32.01
A2	32.60	36.84	33.31	25.15	28.49	27.06	21.74	25.85	30.46	29.27	22.48	33.23	30.30
A3	28.22	34.53	28.69	30.10	30.86	24.31	27.09	22.72	28.12	27.10	22.86	31.82	33.36
C1	21.31	37.43	21.60	22.74	22.88	18.39	21.58	19.68	24.31	20.03	33.43	25.01	20.20
C2	17.87	21.01	17.31	27.61	23.61	22.49	26.31	21.56	18.23	20.06	24.01	16.71	22.21
C3	23.05	17.18	23.39	18.69	17.10	26.25	20.39	25.13	21.49	22.94	28.24	21.30	17.11
U1	18.68	22.84	18.66	27.85	28.05	16.27	27.85	16.00	22.70	21.50	20.86	19.27	28.75
U2	17.06	18.90	16.81	19.07	22.25	28.26	18.98	28.20	16.87	23.64	15.07	17.44	20.23
U3	28.41	15.99	28.13	16.06	16.86	19.91	15.27	20.26	29.17	26.11	22.30	28.23	16.77
G1	24.39	19.98	25.60	22.03	21.74	34.63	24.53	37.30	29.39	29.17	27.64	27.99	19.02
G2	32.80	23.24	32.56	28.16	25.61	22.17	32.96	24.38	34.91	27.02	33.33	32.60	27.15
G3	20.31	32.31	19.78	35.14	35.17	29.51	37.24	31.88	21.21	23.83	22.96	18.64	32.75
GC	46.46	45.5	46.45	51.45	48.71	51.16	54.33	53.32	49.68	47.65	56.31	42.9	46.15
GC1	32.78	36.23	33.66	29.00	30.53	30.53	28.27	35.62	36.02	34.51	36.14	37.25	28.33
GC2	36.13	32.41	32.41	36.13	33.70	34.57	36.35	28.72	35.65	32.94	33.94	34.66	35.66
GC3	31.09	31.36	35.56	34.87	35.76	36.32	35.34	35.64	28.65	32.72	29.91	28.07	36.01

Table 3. Amino acid composition of polyprotein sequences from Flaviviridae

	Amino acid	Dengue virus 1	Dengue virus 2	Dengue virus 3	Japanese encephalitis virus	Murray Valley encephalitis virus	West Nile virus	Tick borne encephalitis virus	Powassan virus	Yellow fever virus	Wesselsbron virus	Hepatitis C virus	Classical swine fever virus	Bovine viral diarrhea virus 1
A	Alanine	7.017	6.812	6.991	8.450	8.416	7.981	8.202	7.760	7.183	7.137	9.199	6.466	5.843
C	Cysteine	1.739	1.681	1.711	1.632	1.660	1.806	1.904	1.816	1.876	1.821	2.868	1.858	1.906
D	Aspartic acid	4.245	3.863	4.012	4.720	4.688	4.515	4.511	4.890	4.632	5.140	4.187	4.710	4.514
E	Glutamic acid	6.515	7.166	6.667	6.031	6.057	6.175	6.678	6.296	6.157	5.374	3.825	6.441	6.795
F	Phenylalanine	3.096	2.978	2.891	3.205	3.262	3.059	2.841	2.723	3.254	3.231	2.868	3.182	3.009
G	Glycine	8.196	8.169	8.348	8.741	8.532	8.535	9.402	9.605	8.854	8.341	8.473	7.510	7.523
H	Histidine	2.152	2.005	2.094	2.098	1.951	1.864	2.402	2.401	2.375	2.144	1.879	2.088	2.106
I	Isoleucine	5.896	6.547	5.988	4.837	5.446	5.098	3.486	3.895	5.277	5.521	4.616	5.448	6.194
K	Lysine	6.073	6.399	6.519	5.536	5.737	5.476	4.628	4.802	5.717	5.962	3.363	7.434	7.447
L	Leucine	9.493	9.112	9.322	8.800	8.969	9.059	9.783	9.810	9.147	8.899	9.891	9.623	9.855
M	Methionine	3.774	3.716	3.658	3.234	2.999	3.321	3.310	3.455	3.811	3.671	2.341	2.240	2.482
N	Asparagine	3.685	3.981	4.012	3.613	3.611	3.641	2.665	2.577	3.635	3.554	2.671	3.742	3.837
P	Proline	4.098	4.276	4.484	4.138	4.193	4.107	3.837	4.100	4.046	4.229	7.056	4.404	4.413
Q	Glutamine	3.243	2.978	3.274	2.535	2.504	2.651	2.695	2.577	2.756	2.526	3.066	3.131	3.260
R	Arginine	5.660	5.751	5.074	2.593	5.999	6.234	7.147	7.145	6.039	5.551	5.605	5.321	5.291
S	Serine	5.955	5.633	5.192	6.119	5.708	5.768	5.712	5.798	6.332	6.432	7.583	4.964	5.191
T	Threonine	7.459	7.815	8.112	5.624	7.164	7.311	6.503	6.120	5.863	6.843	7.319	7.612	7.146
V	Valine	6.692	6.370	6.637	7.139	7.833	8.127	9.168	9.048	8.209	8.253	7.616	8.070	7.447
W	Tryptophan	2.830	2.654	2.802	8.217	2.679	2.709	2.988	2.958	2.492	2.761	2.275	1.629	1.580
Y	Tyrosine	2.182	2.094	2.212	2.739	2.592	2.563	2.138	2.225	2.345	2.614	3.297	4.124	4.162

Table 4. Codon usage data in 13 species of Flaviviridae**.** Dark gray codons are the preferred codons in Flaviviridae. Triplets in bold face indicate a high frequency in coding the amino acid. Light gray codons appear during low frequency coding of the amino acid.

aa	Codon	Dengue1		Dengue 2		Dengue 3		Japanese encephalitis		Murray Valley encephalitis		Human
		1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000
Arg	CGC	6.15	0.06	3.37	0.05	5.60	0.05	8.21	0.09	4.90	0.07	10.68
	AGG	35.80	0.35	15.45	0.25	30.54	0.30	22.44	0.26	18.80	0.27	11.71
	AGA	**36.92**	**0.36**	**25.85**	**0.41**	**41.19**	**0.40**	**26.82**	**0.31**	**20.43**	**0.30**	11.72
	CGG	6.43	0.06	7.59	0.12	7.00	0.07	12.86	0.15	12.26	0.18	11.65
	CGA	8.67	0.08	6.46	0.10	8.41	0.08	10.13	0.12	7.35	0.11	6.24
	CGT	9.79	0.09	3.65	0.06	9.53	0.09	6.02	0.07	4.63	0.07	4.63
Leu	TTG	8.95	0.16	**20.23**	**0.26**	7.28	0.13	**22.44**	**0.28**	**34.87**	**0.35**	12.75
	TTA	4.20	0.08	12.36	0.16	5.04	0.09	8.76	0.11	11.44	0.11	7.43
	CTG	9.51	0.17	15.17	0.19	12.05	0.22	19.16	0.24	23.97	0.24	40.13
	CTA	6.71	0.12	10.96	0.14	5.60	0.10	7.94	0.10	8.72	0.09	7.04
	CTT	**15.10**	**0.28**	10.12	0.13	**17.37**	**0.32**	12.04	0.15	10.90	0.11	13.01
	CTC	10.35	0.19	10.12	0.13	7.57	0.14	10.67	0.13	10.08	0.10	19.67
Ser	AGT	26.01	0.28	7.31	0.09	23.82	0.27	9.30	0.09	9.81	0.12	12.05
	AGC	**30.49**	**0.33**	7.87	0.10	**32.78**	**0.37**	12.04	0.12	11.71	0.15	19.45
	TCG	3.08	0.03	8.71	0.11	1.12	0.01	18.88	0.18	7.90	0.10	4.47
	TCA	10.35	0.11	**29.22**	**0.36**	9.53	0.11	**34.76**	**0.33**	**26.42**	**0.33**	12.00
	TCT	13.43	0.14	11.24	0.14	11.21	0.13	13.14	0.13	10.62	0.13	14.89
	TCC	9.79	0.11	15.73	0.20	10.65	0.12	15.87	0.15	13.89	0.17	17.63
Ala	GCG	2.80	0.08	5.06	0.16	3.08	0.08	8.76	0.17	8.99	0.19	7.55
	GCA	8.39	0.23	**14.89**	**0.48**	**15.97**	**0.39**	**19.43**	**0.38**	**19.89**	**0.42**	16.00
	GCT	**18.46**	**0.50**	6.74	0.22	13.45	0.33	12.59	0.25	8.72	0.18	18.57
	GCC	6.99	0.19	4.50	0.14	8.13	0.20	9.85	0.19	9.81	0.21	28.28
Gly	GGG	14.27	0.18	15.17	0.28	17.65	0.21	**24.08**	**0.35**	15.80	0.26	16.48
	GGA	**31.61**	**0.40**	**21.35**	**0.40**	**31.94**	**0.37**	21.62	0.32	**25.88**	**0.43**	16.42
	GGT	14.55	0.18	8.99	0.17	18.21	0.21	10.67	0.16	10.62	0.18	10.80
	GGC	19.58	0.24	8.43	0.16	17.93	0.21	11.49	0.17	7.90	0.13	22.56
Pro	CCG	3.36	0.08	7.59	0.15	2.24	0.05	13.68	0.20	10.62	0.17	7.04
	CCA	**17.34**	**0.39**	**23.04**	**0.46**	**16.25**	**0.38**	**30.38**	**0.45**	**32.42**	**0.51**	16.84
	CCT	14.83	0.33	8.43	0.17	14.85	0.35	11.77	0.17	11.44	0.18	17.42
	CCC	8.95	0.20	11.52	0.23	9.25	0.22	12.04	0.18	9.26	0.15	20.03
Thr	ACG	6.15	0.10	8.71	0.14	5.88	0.10	16.15	0.22	10.62	0.16	6.15
	ACA	18.46	0.30	**28.66**	**0.45**	13.45	0.23	**29.28**	**0.39**	**29.69**	**0.45**	14.91
	ACT	**20.70**	**0.34**	12.64	0.20	**19.89**	**0.35**	13.68	0.18	12.80	0.19	13.01
	ACC	15.66	0.26	13.49	0.21	18.21	0.32	15.87	0.21	13.08	0.20	19.09
Val	GTG	10.63	0.27	**14.89**	**0.44**	11.21	0.28	**15.60**	**0.45**	**19.61**	**0.52**	28.56
	GTA	5.31	0.13	7.59	0.23	4.48	0.11	6.29	0.18	5.18	0.14	7.06
	GTT	**15.38**	**0.39**	7.02	0.21	**16.25**	**0.40**	7.94	0.23	6.27	0.17	10.98
	GTC	8.11	0.21	4.21	0.13	8.69	0.21	5.20	0.15	6.54	0.17	14.63
Asn	AAT	**28.81**	**0.52**	12.36	0.39	28.02	0.48	9.30	0.37	11.71	0.43	16.72
	AAC	26.29	0.48	**19.67**	**0.61**	**30.26**	**0.52**	**15.60**	**0.63**	**15.53**	**0.57**	19.17
Asp	GAT	**22.38**	**0.54**	10.40	0.50	**21.29**	**0.57**	**9.58**	**0.60**	**9.81**	**0.54**	21.98
	GAC	18.74	0.46	10.40	0.50	16.25	0.43	6.29	0.40	8.44	0.46	25.50
Cys	TGT	**16.22**	**0.54**	**15.17**	**0.53**	**15.13**	**0.51**	14.50	0.40	17.16	0.47	10.31
	TGC	13.99	0.46	13.49	0.47	14.57	0.49	**22.17**	**0.60**	**19.61**	**0.53**	12.55
His	CAT	**33.29**	**0.58**	17.42	0.46	**32.50**	**0.59**	9.58	0.42	13.35	0.48	10.69
	CAC	24.34	0.42	**20.51**	**0.54**	22.98	0.41	**13.14**	**0.58**	**14.44**	**0.52**	15.03
Phe	TTT	**13.43**	**0.59**	**11.52**	**0.54**	**14.85**	**0.58**	7.94	0.38	**13.25**	**0.56**	17.16
	TTC	9.51	0.41	9.83	0.46	10.93	0.42	**12.86**	**0.62**	10.35	0.44	20.39
Tyr	TAT	**8.67**	**0.61**	8.43	0.47	**10.37**	**0.54**	4.93	0.50	5.18	0.44	12.09
	TAC	5.59	0.39	**9.55**	**0.53**	8.97	0.46	4.93	0.50	**6.54**	**0.56**	15.41
Gln	CAG	15.94	0.42	**41.30**	**0.57**	16.25	0.36	**30.10**	**0.60**	**33.78**	**0.62**	34.39
	CAA	**22.38**	**0.58**	31.19	0.43	**28.58**	**0.64**	19.70	0.40	20.70	0.38	12.03
Glu	GAG	14.83	0.32	29.22	0.49	15.97	0.31	**27.64**	**0.54**	**33.23**	**0.62**	39.98
	GAA	**31.89**	**0.68**	**30.91**	**0.51**	**35.58**	**0.69**	23.26	0.46	20.70	0.38	28.92
Lys	AAG	23.22	0.40	**42.43**	**0.53**	17.93	0.36	**29.56**	**0.60**	**31.60**	**0.55**	32.22
	AAA	**34.13**	**0.60**	38.21	0.47	**32.50**	**0.64**	19.70	0.40	26.15	0.45	24.04
Ile	ATA	6.99	0.19	**10.96**	**0.39**	6.16	0.19	5.75	0.24	7.90	0.27	7.28
	ATT	13.15	0.36	8.43	0.30	**14.57**	**0.46**	7.66	0.32	**11.99**	**0.41**	15.79
	ATC	**15.94**	**0.44**	8.99	0.32	11.21	0.35	**10.67**	**0.44**	9.26	0.32	21.07

aa	Codon	West Nile		Tick-borne encephalitis		Powassan_virus		Yellow fever virus		Wesselsbron_virus		Human
		1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000
Arg	CGC	9.52	0.14	7.30	0.08	10.24	0.15	8.56	0.11	5.83	0.07	10.68
	AGG	15.78	0.24	**32.18**	**0.34**	16.88	0.24	24.03	0.30	24.97	0.29	11.71
	AGA	**20.95**	**0.32**	24.88	0.26	**20.20**	**0.29**	**24.86**	**0.31**	**30.24**	**0.35**	11.72
	CGG	8.16	0.12	18.66	0.20	9.41	0.13	9.12	0.12	8.60	0.10	11.65
	CGA	5.98	0.09	6.22	0.07	6.37	0.09	5.25	0.07	6.38	0.07	6.24
	CGT	5.71	0.09	6.22	0.07	7.20	0.10	7.18	0.09	9.71	0.11	4.63
Leu	TTG	21.49	0.24	**30.29**	**0.36**	23.25	0.24	8.01	0.12	12.21	0.19	12.75
	TTA	4.08	0.05	4.87	0.06	2.77	0.03	5.25	0.08	6.94	0.11	7.43
	CTG	**27.20**	**0.30**	22.71	0.27	**27.95**	**0.29**	12.15	0.19	11.10	0.17	40.13
	CTA	9.79	0.11	5.41	0.06	8.30	0.09	7.73	0.12	8.60	0.13	7.04
	CTT	10.34	0.11	7.84	0.09	17.16	0.18	**16.57**	**0.26**	**16.65**	**0.25**	13.01
	CTC	17.68	0.20	12.44	0.15	16.33	0.17	14.64	0.23	9.99	0.15	19.67
Ser	AGT	12.51	0.21	12.17	0.12	11.90	0.20	**23.76**	**0.29**	**24.14**	**0.27**	12.05
	AGC	13.06	0.22	14.60	0.15	**13.84**	**0.23**	18.78	0.23	22.48	0.25	19.45
	TCG	5.71	0.10	11.90	0.12	5.54	0.09	3.04	0.04	4.16	0.05	4.47
	TCA	**14.96**	**0.25**	**27.31**	**0.28**	12.73	0.21	9.12	0.11	7.49	0.08	12.00
	TCT	6.53	0.11	13.79	0.14	5.54	0.09	15.19	0.19	15.82	0.18	14.89
	TCC	5.98	0.10	18.66	0.19	10.52	0.18	12.15	0.15	15.26	0.17	17.63
Ala	GCG	9.79	0.13	10.55	0.19	10.79	0.14	4.14	0.09	5.55	0.12	7.55
	GCA	19.04	0.25	**20.55**	**0.37**	19.65	0.25	14.09	0.30	12.76	0.28	16.00
	GCT	**25.30**	**0.33**	12.98	0.23	21.04	0.27	**18.78**	**0.40**	**18.31**	**0.40**	18.57
	GCC	22.85	0.30	11.63	0.21	**26.02**	**0.34**	9.67	0.21	9.43	0.20	28.28
Gly	GGG	14.96	0.18	23.80	0.30	24.91	0.25	16.30	0.16	15.26	0.18	16.48
	GGA	**42.17**	**0.50**	**29.20**	**0.37**	**39.58**	**0.41**	**35.08**	**0.34**	**28.02**	**0.32**	16.42
	GGT	9.25	0.11	11.36	0.14	12.73	0.13	26.80	0.26	22.75	0.26	10.80
	GGC	17.41	0.21	14.87	0.19	20.48	0.21	24.86	0.24	20.26	0.23	22.56
Pro	CCG	4.62	0.11	11.36	0.17	5.26	0.13	5.80	0.10	8.05	0.17	7.04
	CCA	**19.04**	**0.45**	**24.34**	**0.37**	**17.16**	**0.41**	19.06	0.33	**15.54**	**0.33**	16.84
	CCT	8.71	0.20	15.95	0.24	7.75	0.19	**22.10**	**0.38**	13.87	0.29	17.42
	CCC	10.34	0.24	13.52	0.21	11.62	0.28	11.33	0.19	9.71	0.21	20.03
Thr	ACG	11.43	0.16	11.90	0.17	13.29	0.21	5.80	0.15	7.49	0.18	6.15
	ACA	22.58	0.31	**28.12**	**0.40**	**19.93**	**0.32**	**11.05**	**0.29**	11.65	0.28	14.91
	ACT	15.23	0.21	14.06	0.20	11.90	0.19	**11.05**	**0.29**	**13.04**	**0.31**	13.01
	ACC	**22.85**	**0.32**	16.50	0.23	16.88	0.27	9.94	0.26	9.43	0.23	19.09
Val	GTG	**37.81**	**0.46**	**23.53**	**0.60**	**42.35**	**0.48**	14.36	0.33	8.60	0.22	28.56
	GTA	6.80	0.08	3.52	0.09	8.03	0.09	5.25	0.12	6.94	0.17	7.06
	GTT	15.78	0.19	5.14	0.13	18.27	0.21	**15.19**	**0.34**	**15.26**	**0.38**	10.98
	GTC	21.22	0.26	6.76	0.17	19.65	0.22	9.39	0.21	9.16	0.23	14.63
Asn	AAT	13.33	0.37	6.49	0.33	11.62	0.46	**19.34**	**0.58**	21.09	0.50	16.72
	AAC	**22.85**	**0.63**	**12.98**	**0.67**	**13.84**	**0.54**	13.81	0.42	20.81	0.50	19.17
Asp	GAT	20.40	0.47	5.95	0.28	18.82	0.39	**23.48**	**0.55**	**21.09**	**0.55**	21.98
	GAC	**23.39**	**0.53**	**15.68**	**0.73**	**29.06**	**0.61**	19.06	0.45	16.93	0.45	25.50
Cys	TGT	8.43	0.45	16.50	0.41	**11.62**	**0.60**	**23.20**	**0.57**	**23.03**	**0.52**	10.31
	TGC	**10.34**	**0.55**	**23.53**	**0.59**	7.75	0.40	17.40	0.43	21.64	0.48	12.55
His	CAT	7.62	0.38	7.03	0.35	**12.73**	**0.51**	**35.91**	**0.65**	**36.63**	**0.59**	10.69
	CAC	**12.51**	**0.62**	**13.25**	**0.65**	12.18	0.49	19.61	0.35	25.25	0.41	15.03
Phe	TTT	13.06	0.45	7.57	0.39	**13.84**	**0.51**	**14.09**	**0.56**	**15.82**	**0.56**	17.16
	TTC	**16.05**	**0.55**	**11.63**	**0.61**	13.56	0.49	11.05	0.44	12.21	0.44	20.39
Tyr	TAT	9.52	0.38	3.24	0.44	9.41	0.45	**8.56**	**0.58**	**9.43**	**0.58**	12.09
	TAC	**15.23**	**0.62**	**4.06**	**0.56**	**11.62**	**0.55**	6.08	0.42	6.94	0.42	15.41
Gln	CAG	**14.69**	**0.54**	**25.15**	**0.58**	**17.71**	**0.65**	19.89	0.41	14.71	0.34	34.39
	CAA	12.51	0.46	18.39	0.42	9.41	0.35	**28.18**	**0.59**	28.02	0.66	12.03
Glu	GAG	**31.01**	**0.52**	**32.45**	**0.65**	**34.32**	**0.56**	20.44	0.36	13.60	0.35	39.98
	GAA	29.11	0.48	17.31	0.35	27.40	0.44	**37.02**	**0.64**	**25.80**	**0.65**	28.92
Lys	AAG	**31.83**	**0.58**	**21.36**	**0.56**	**24.91**	**0.51**	14.92	0.39	15.54	0.33	32.22
	AAA	22.85	0.42	17.04	0.44	24.36	0.49	**23.48**	**0.61**	**31.08**	**0.67**	24.04
Ile	ATA	11.15	0.22	5.41	0.29	8.58	0.23	6.35	0.25	5.27	0.19	7.28
	ATT	**21.22**	**0.43**	**6.49**	**0.35**	11.07	0.30	**10.50**	**0.41**	9.43	0.34	15.79
	ATC	17.41	0.35	**6.49**	**0.35**	**17.71**	**0.47**	8.56	0.34	**13.32**	**0.48**	21.07

aa	Codon	Hepatitis C virus		Classical swine fever virus		Bovine viral diarrhea virus 1		Human
		1/1000	Fract	1/1000	Fract	1/1000	Fract	1/1000
Arg	CGC	20.99	0.19	6.34	0.06	2.70	0.03	10.68
	AGG	16.54	0.15	27.82	0.27	27.50	0.35	11.71
	AGA	12.72	0.12	**36.12**	**0.35**	**28.97**	**0.37**	11.72
	CGG	**22.58**	**0.21**	10.74	0.10	8.84	0.11	11.65
	CGA	17.18	0.16	12.45	0.12	6.87	0.09	6.24
	CGT	18.45	0.17	10.49	0.10	3.19	0.04	4.63
Leu	TTG	10.18	0.11	7.81	0.11	**21.85**	**0.27**	12.75
	TTA	7.95	0.09	9.52	0.14	13.75	0.17	7.43
	CTG	**21.95**	**0.24**	12.20	0.18	20.87	0.26	40.13
	CTA	9.22	0.10	13.91	0.20	13.50	0.17	7.04
	CTT	**22.58**	**0.24**	**19.03**	**0.27**	7.12	0.09	13.01
	CTC	20.67	0.22	6.83	0.10	3.68	0.05	19.67
Ser	AGT	15.90	0.20	**25.87**	**0.36**	10.80	0.14	12.05
	AGC	**21.31**	**0.27**	21.96	0.31	14.73	0.19	19.45
	TCG	9.22	0.12	0.98	0.01	7.86	0.10	4.47
	TCA	7.95	0.10	7.08	0.10	**19.40**	**0.25**	12.00
	TCT	12.72	0.16	10.25	0.14	12.77	0.16	14.89
	TCC	13.04	0.16	5.37	0.08	12.77	0.16	17.63
Ala	GCG	13.99	0.22	1.95	0.05	5.65	0.16	7.55
	GCA	13.99	0.22	11.96	0.30	**16.45**	**0.46**	16.00
	GCT	**22.58**	**0.35**	9.03	0.23	6.14	0.17	18.57
	GCC	13.99	0.22	**16.59**	**0.42**	7.37	0.21	28.28
Gly	GGG	**27.99**	**0.28**	17.81	0.18	**23.82**	**0.33**	16.48
	GGA	24.49	0.25	**30.99**	**0.32**	21.85	0.30	16.42
	GGT	20.99	0.21	26.11	0.27	13.50	0.19	10.80
	GGC	25.45	0.26	22.69	0.23	12.52	0.17	22.56
Pro	CCG	12.40	0.15	1.46	0.03	11.05	0.20	7.04
	CCA	19.72	0.23	17.33	0.31	**23.57**	**0.42**	16.84
	CCT	**26.40**	**0.31**	**20.99**	**0.38**	10.07	0.18	17.42
	CCC	**26.08**	**0.31**	15.86	0.29	11.78	0.21	20.03
Thr	ACG	7.00	0.15	1.71	0.04	9.82	0.13	6.15
	ACA	12.72	0.26	15.86	0.33	**28.73**	**0.37**	14.91
	ACT	13.68	0.28	**16.59**	**0.34**	17.43	0.23	13.01
	ACC	**14.63**	**0.30**	14.15	0.29	21.36	0.28	19.09
Val	GTG	14.31	0.25	9.27	0.21	**15.71**	**0.39**	28.56
	GTA	11.45	0.20	11.71	0.26	12.77	0.31	7.06
	GTT	**17.18**	**0.30**	**16.59**	**0.37**	7.12	0.17	10.98
	GTC	14.95	0.26	6.83	0.15	5.16	0.13	14.63
Asn	AAT	**10.81**	**0.53**	15.62	0.44	12.52	0.44	16.72
	AAC	9.54	0.47	**20.01**	**0.56**	**16.20**	**0.56**	19.17
Asp	GAT	14.95	0.49	**25.62**	**0.55**	4.91	0.42	21.98
	GAC	**15.59**	**0.51**	21.23	0.45	**6.87**	**0.58**	25.50
Cys	TGT	20.36	0.46	**14.15**	**0.53**	**14.24**	**0.52**	10.31
	TGC	**23.54**	**0.54**	12.69	0.47	13.01	0.48	12.55
His	CAT	23.85	0.44	22.45	0.50	**13.01**	**0.51**	10.69
	CAC	30.85	0.56	22.69	0.50	12.28	0.49	15.03
Phe	TTT	9.54	0.45	**12.45**	**0.70**	**8.59**	**0.66**	17.16
	TTC	**11.45**	**0.55**	5.37	0.30	4.42	0.34	20.39
Tyr	TAT	10.18	0.48	12.45	0.47	14.73	0.48	12.09
	TAC	**10.81**	**0.52**	**13.91**	**0.53**	**15.71**	**0.52**	15.41
Gln	CAG	17.49	0.42	22.21	0.39	**30.69**	**0.57**	34.39
	CAA	**23.85**	**0.58**	**35.14**	**0.61**	22.83	0.43	12.03
Glu	GAG	8.27	0.34	19.77	0.38	**17.43**	**0.57**	39.98
	GAA	**16.22**	**0.66**	**31.72**	**0.62**	13.01	0.43	28.92
Lys	AAG	5.41	0.35	13.67	0.35	**32.65**	**0.52**	32.22
	AAA	**10.18**	**0.65**	**24.89**	**0.65**	30.69	0.48	24.04
Ile	ATA	**9.54**	**0.43**	9.27	0.27	20.38	0.48	7.28
	ATT	5.73	0.26	**17.08**	**0.50**	11.54	0.27	15.79
	ATC	7.00	0.31	8.05	0.23	10.56	0.25	21.07

Table 5. Genome signature of various genera of Flaviviridae.

Genus	Subgenus	*Species*	*Nucleotide signature*	
			High	low
Flavivirus	Dengue virus group	Dengue virus 1 Dengue virus 2 Dengue virus 3	**T,C** **A**	 **T,C**
	Japanese encephalitis virus group	Japanese encephalitis virus Species: Murray Valley encephalitis virus West_Nile_virus	A,G	T,C
	Yellow fever virus group	Yellow fever virus Wesselsbron virus		
	Tick-borne encephalitis virus group	Tick-borne encephalitis virus Powassan virus	G	T,C
Hepacivirus		Hepatitis C virus	C,G	A,T
Pestivirus		Classical swine fever virus Bovine viral diarrhea virus 1	A	C

Discussion

Flaviviruses are transmitted by mosquitoes, ticks, or directly between vertebrate hosts. It was demonstrated (Jenkins et al., 2001) that those viruses associated with ticks have a significantly lower G+C content than non-vector-borne flaviviruses. In contrast, mosquito-borne viruses had an intermediate G+C content which was not significantly different from those of the other two groups.

It has been proposed by Wright (1990) that most variable ENC is a more direct measure of synonymous codon bias. The ENC values of different Flaviviridae genes vary from 51.84 to 58.65, with a mean of 54.004 and S.D. of 1.850. We found that all the ENC values of these strains were more than 50. The ENC values range from 20 to 61. In an extremely biased gene where only one codon is used for each amino acid, this value would be 20; in an unbiased gene, it would be 61. We conclude that there is not much codon usage bias in Flaviviridae genome.

It has already been shown that Cysteine (Lobry et al., 1994; Rodrı´guez-Maseda et al., 1994; Musto et al., 1997) and Leucine are the least and the most frequent amino acids among all of the species of Flaviviridae. *Hepacivirus* genus and *Tick-borne encephalitis* virus group subgenus show major exceptions at amino acid composition in polyprotein sequences. For example, Glutamic acid and Lysine are the lowest number in *Hepacivirus* genus while have the highest number in other species. CGN and AGN codons encode Arginine. *Tick-borne encephalitis* virus group subgenus with the highest amount of G has the highest number of Arginine residue. This group also has a lesser amount of Asparagine, Isoleucine, Lysine and Phenylalanine whilst has higher number of Valine, Arginine and Glycine residues. Moreover, it is observed that Tryptophan has a maximum in *Japanese encephalitis* virus species and a minimum number in *Pestivirus* genus. Also in *Pestivirus* genus, Tyrosine has the highest number between all of the species.

We have found that Arg, Leu and Ser have six-fold coding degeneracy. All the examined species, excluding *Hepatitis C* virus, use AGA for Arg most frequently, while *Hepatitis C* virus most commonly uses CGG, CGC, CGA, CGT and CGG codons are less frequent in the majority of members. The most and the least commonly used codons for Leu and Ser are different.

Ala, Gly, Pro, Thr and Val have four fold coding degeneracy. For coding Ala, GCG is used less frequently while GCA and GCT are used more in

many of the species. For Gly, *Japanese encephalitis*, *Hepatitis C* virus, and *Bovine viral diarrhea* virus mostly use GGG, while other species mostly use GGA. ACG (for Thr) and CCG (for Pro) have the lowest frequency in most of species. GTG is used for Val most often in *Bovine viral diarrhea* virus, *West Nile* virus, *Tick-borne encephalitis* virus, *Powassan* virus, *Dengue 2* virus, *Japanese encephalitis* virus and *Murray Valley encephalitis* virus while in other species GTT is mainly used.

Asn, Asp, Cys, His, Phe and Tyr have two-fold codon degeneracy. Mostly for Asn, Cys and Phe, the usage of codons with T ending is higher than codons with C ending. For Asp, Gln, Glu, Lys and His, the usage of two codons is approximately the same. Ile is the only amino acid which has three-fold codon degeneracy. ATA, ATT and ATC are the least commonly used synonymous codons in the members of *Flavivirus*, *Hepacivirus*, and *Pestivirus* genus, respectively. Based on our findings, *Flavivirus genus* can be classified in four subgenuses.

We have analyzed the codon usage, base and amino acid compositions of 13 species of Flaviviridae. Results reveal that there is not much codon usage bias in Flaviviridae genome. In the future studies, analysis of different factors, affecting codon usage variation, can increase our knowledge about processes involved in the Flaviviridae evolution and the selective forces that significantly influence codon usage bias.

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Changes in anti-oxidant activity of *Thymus transcaspicus* (Klokov) during growth and developmental stages

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Abstract

Antioxidant activities protect the cell against oxidative agents that are constant metabolic by-products. The aim of this study was to investigate the relationship between harvesting time of *Thymus transcaspicus* and its antioxidant activities. The plant samples were harvested 5 times in different growth phases from 17 April to 22 July 2008, and its antioxidant activity was studied using the ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, and β-carotene bleaching (BCB) assays. The results of FRAP assay indicated that the reduction activity of the plant was in the highest level in stage 5 of sampling. The result of DPPH assay showed that the crude extract of the plant was more capable of DPPH radical scavenging in stage 2. The highest level of gallic acid and quercetin in the crude extract of *T. transcaspicus* was determined as 85.29 ± 6.22 mg and 18.88 ± 0.9 mg in stage 2, respectively. Therefore, stage 2 was the optimum time to harvest the *T. transcaspicus*.

Keywords**:** *Thymus transcaspicus,* antioxidant capacity, growth stages, total phenolic content, total flavonoid content

Introduction

The genus *Thymus* L. (*Lamiaceae*), an aromatic and medicinal plant, includes numerous species with quite different botanical characteristics. This genus consists of about 215 species of herbaceous perennials and sub shrubs that grow naturally in the Mediterranean region, and is represented in the Iranian flora by 14 species (Javadi et al., 2009). Leaves and flowers of *Thymus* species are commonly used as herbal tea, spice, flavoring agents and medicinal plants. Furthermore, *Thymus* oils and extracts are widely used in pharmaceutical, cosmetic and perfume industries and are also used for flavoring and preserving food products. It has been reported that *Thymus* species have strong antibacterial, antifungal, antiviral, anti-parasitic, spasmolytic and antioxidant activities (Imelouane et al., 2009). *Thymus transcaspicus* (Klokov; Lamiaceae family), is an endemic species that exists in the Northern regions of Iran.

Epidemiological studies have indicated possible roles of fruits and vegetables in preventing numerous diseases such as cancer, cardiovascular disorders, cataract, neuro-degenerative diseases, atherosclerosis and inflammation. The preservative effects of many plant spices are attributed to the large amounts of antioxidative constituents in their tissues (Deepa et al., 2007). Antioxidant compounds, are important in protecting plants against harmful chemical compounds including free radicals and reactive oxygen species (ROS) that are constantly produced by the cell metabolism and their concentration increases under stress situations (Ferreyra et al., 2007). Although free radicals and ROS can rapidly attack all types of biomolecules and lead to lipid peroxidation, plasma membrane injury and proteins and deoxyribonucleic acid damages that finally end in cell death (Berlett and Stadtman, 1997), antioxidants are able to scavenge or deactivate free radicals before they attack plant cells. The antioxidant systems consist of enzymatic (superoxide dismutase, catalase, peroxidase, etc.) and non-enzymatic components (carotenoids, phenolic compounds e.g. catechins, anthocyanins and vitamins A, C, and E). The level and mode of antioxidant activity depends on genotype, maturity stages, and conditions of plant growth (Chirinos et al., 2007). Changes in antioxidant activity or antioxidant compounds during plant growth and developmental stages appear to vary among fruits, vegetables and herbs, even though there is no consistent report. Prior et al. (1998) found an increase in antioxidant activity and total phenols

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and anthocyanins contents in ripe fruit of industrial berry varieties. Also, Howard et al. (2000) observed that upon *Capsicum* cultivars growth and maturation, the concentration of antioxidant constituents was increased. Wang and Lin (2000) found that blackberries and strawberries had the highest antioxidant values during the green stages, whereas red raspberries had the highest antioxidant activity at the ripe stage. Moreover, it has been reported that cherry tomatoes harvested at full ripeness exhibited the highest level of carotenoids and antioxidant activity in the water-insoluble fraction (Raffo et al., 2002). Huang et al., (2007) observed that the activity of oxygen-scavenging enzymes (SOD, CAT and G-POD) was decreased with ripening and maturation of fruit in orange pulp. Furthermore, the highest antioxidant activity was observed at the stage of flower opening of the plant (Fu et al., 2009). However, a decreasing trend of antioxidant activity in blueberry (*Vaccinium corymbosum* L.) fruit during ripening was observed by Castrejón et al. (2008). Alteration in antioxidant activity and related compounds during growth and maturation affects functional properties in plants for their narrow harvest window (Fu et al., 2008).

To our knowledge, there is no available information on the variations in antioxidant properties of *T. transcaspicus* during different growth and developmental stages. Therefore, the aim of this research is to study the antioxidant activity of *T. transcaspicus* at different developmental stages, in order to determine the best harvesting time of this plant.

Materials and Methods

Collection and preparation of plant samples

Fresh aerial parts of *T. transcaspicus* were collected from Sar-Aliabad with a longitude of 54° 33' 11" N. and a latitude of 36° 40' 0" E. and an altitude of 2339 ± 10.62 meter around Gorgan city of Iran, during the early and late of growth (early of flowering), mid (with 70% flowering) and late (early of seed formation) of flowering, and seed formation (100%) stages with 24 days intervals (17 Apr – 22 Jul, 2008).

One part of the samples were frozen with liquid nitrogen for ferric reducing antioxidant power (FRAP) assay, and the ascorbic acid content was then stored in -70°C (up to 3 days). Other samples were dried at room temperature and powdered. One gram of each powder sample was extracted in 50 ml of 80% methanol by maceration (48 h). The extract was then filtered and the pellet was dissolved in 80% methanol and remained for 24 hours re-extracted. Finally, both filtered solutions were mixed and concentrated using rotary evaporator at 40°C, and the crude extract was stored at 4°C until usage (up to 10 days).

Total phenolic and flavonoid contents

Total phenolic content of the crude extracts of *T. transcaspicus* was determined by Folin–Ciocalteu reagent (Ercisli and Orhan, 2007), and expressed as milligram gallic acid equivalents (GAE)/$g^{-1}dw^{-1}$. The total flavonoid content of the extracts was determined using the aluminum chloride colorimetric method described by Chang, et al., (2002). The same procedure was repeated for all standard quercetin solutions (20–200 mg/l) to obtain a curve. The data were expressed as milligram quercetin equivalents (QE)/$g^{-1}dw^{-1}$.

Ascorbic acid content

Ascorbic acid (ASA) content was determined by 2, 6-dichlorophenolindophenol-dye method as described by Deepa et al. (2007), and the results were expressed as mg ASA/100 g fw.

Antioxidant activity assays

FRAP assay

The FRAP assay of the extracts was assessed on the basis of the ferric to ferrous ion reduction (Benzie and Strain 1996). The standard curve was obtained based on the absorbance of several concentrations of freshly prepared ammonium ferrous sulphate (100–1000 µM ferrous ion). Tests were carried out in triplicates and expressed as mmol Fe^{2+} g^{-1} fw.

1, 1-diphenyl-2-picrylhydrazyl free radical scavenging activity

The ability of corresponding extracts and some pure compounds in donating a hydrogen atom or electron was determined by bleaching purple-colored methanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. In this spectrophotometric assay, the stable DPPH radical is used as the reagent according to a procedure described by Cuendet et al., (1997). Tests were carried out in triplicates and results were expressed as inhibition of free radical by DPPH in percent (%I), and calculated by the following:

$$\%I = (A_{blank} - A_{sample})/A_{blank} \times 100$$

Where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the sample. $IC_{50,}$ which denotes the amount (mg) of plant extract that inhibits DPPH radicals by 50%, was calculated from the plotted graph of inhibition

percentage against extract concentration. The ascorbic acid (AA) was used as a standard and results were expressed as ascorbic acid equivalent antioxidant activity (AEAC) using the following equation:

$$AEAC\ (mg\ AA/gdw) = IC_{50}\ (ascorbate)/IC_{50}\ (sample) \times 100$$

β-carotene bleaching (BCB) assay

BCB assay determines antioxidant capacity by measuring the inhibition of volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius, et al., 1998). The degradation rate (DR) of β-carotene was calculated as $\ln (A_{initial} / A_{60})/60$ and the percentage of antioxidant activity (AOA%) was calculated as:

$$[(DR_{control} - DR_{sample})/DR_{control}] \times 100$$

Results

Ascorbic acid content

As observed in table 1, ascorbic acid content significantly varied among samples harvested during different stages and it ranged from 2.01 ± 0.29 mg/100 g fw in stage 2 to 5.81 ± 0.35 mg/100 g fw in stage 5.

Total phenolic and flavonoid contents

As it is summarized in table 1, the stage of harvesting affected the total phenolic and flavonoid contents of plant samples. The total phenol content in samples varied from 35.75 ± 3.15 mg GAE/g dw, to 85.29 ± 6.22 mg GAE/g dw, and the highest content of the total phenol was determined in stage 2 of sampling. The content of flavonoids ranged from 7.54 mg ± 0.43 QE/g dw in stage 4 to 18.88 ± 0.9 mg QE/g dw in stage 2.

Antioxidant activity

Since different antioxidant compounds may act through different mechanisms *in vivo*, no single method can fully evaluate the antioxidant activity of bio-samples. Therefore, three methods, FRAP, DPPH, and BCB assays, were used in the present study to evaluate the antioxidant activity of plant extracts. FRAP, DPPH and BCB assays evaluate the reducing activities (Benzie and Strain, 1996), hydrogen atom-or-electron donation ability (Cuendet et al., (1997), and the inhibition lipid peroxidation (Lim and Quah, 2007), respectively.

The results of FRAP and BCB revealed that the crude extract of *T. transcaspicus* in stage 5 of growth had the highest anti-oxidant activity (figures 1 and 2). However, DPPH assay indicated that in stage 2 of harvesting, the highest antioxidant activity was observed (figure 3). Based on these three methods, the lowest anti-oxidant activity has been determined in stages 1, 4 and 3 (figures 1, 2 and 3). Our results have also indicated that the ascorbic acid content in *T. transcaspicus* was correlated with ferric reducing antioxidant activity (figure 4. a, $R^2 = 0.67$). Present findings showed that the phenol and flavonoid content in *T. transcaspicus* were lineary correlated with DPPH free radical scavenging activity ($R^2 = 0.69$, $R^2 = 0.80$, respectively), (figures 4b and 4c).

Table 1. Total phenolic and flavonoid contents of *T. transcaspicus* in different stages of sampling.

Stage of sampling	Date	Phenology	Total phenol (mgGAE/gdw)	Total flavonoid (mgQE/gdw)	Ascorbic acid content (mg/100gfw)
1	2008/4/17	Early growth (two weeks after growth)	35.96 ± 4.97 ^{c}	10.71 ± 0.89 ^{b}	3.39 ± 0.34 ^{c}
2	2008/5/12	Late growth (early flowering)	85.29 ± 6.22 ^{a}	18.88 ± 0.9 ^{a}	2.01 ± 0.29 ^{e}
3	2008/6/5	Mid flowering (with 70% flowering)	68.31 ± 4.99 ^{b}	10.34 ± 0.33 ^{b}	3.88 ± 0.32 ^{b}
4	2008/6/29	Late flowering (early seed formation)	35.75 ± 3.15 ^{c}	7.54 ± 0.43 ^{d}	2.85 ± 0.40 ^{d}
5	2008/7/22	Seed formation (100%)	37.03 ± 2.14 ^{c}	8.91 ± 0.74 ^{c}	5.81 ± 0.35 ^{a}

Data are expressed as means ± S.D. (n=8). Similar upper case letters indicate no significant differences (Duncan test, $P<0.05$).

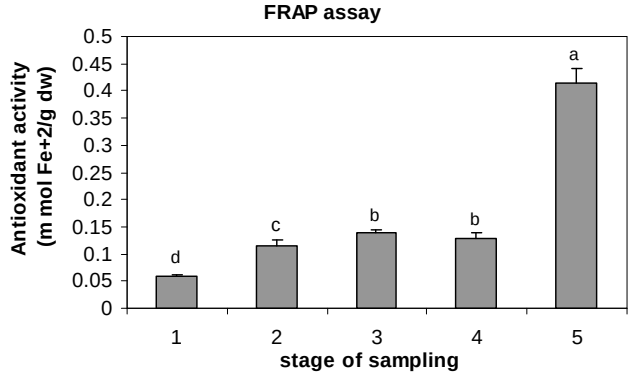

Figure 1. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by FRAP assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

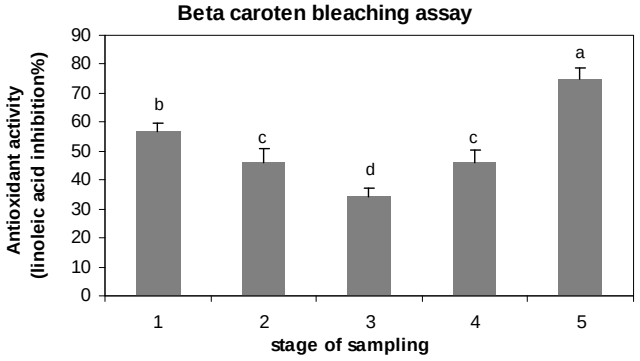

Figure 2. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by BCB assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

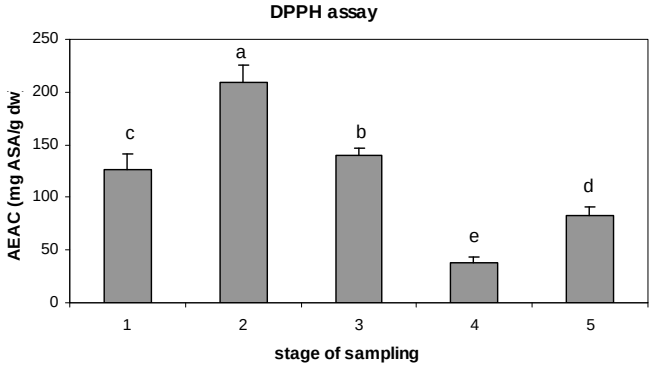

Figure 3. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by DPPH assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

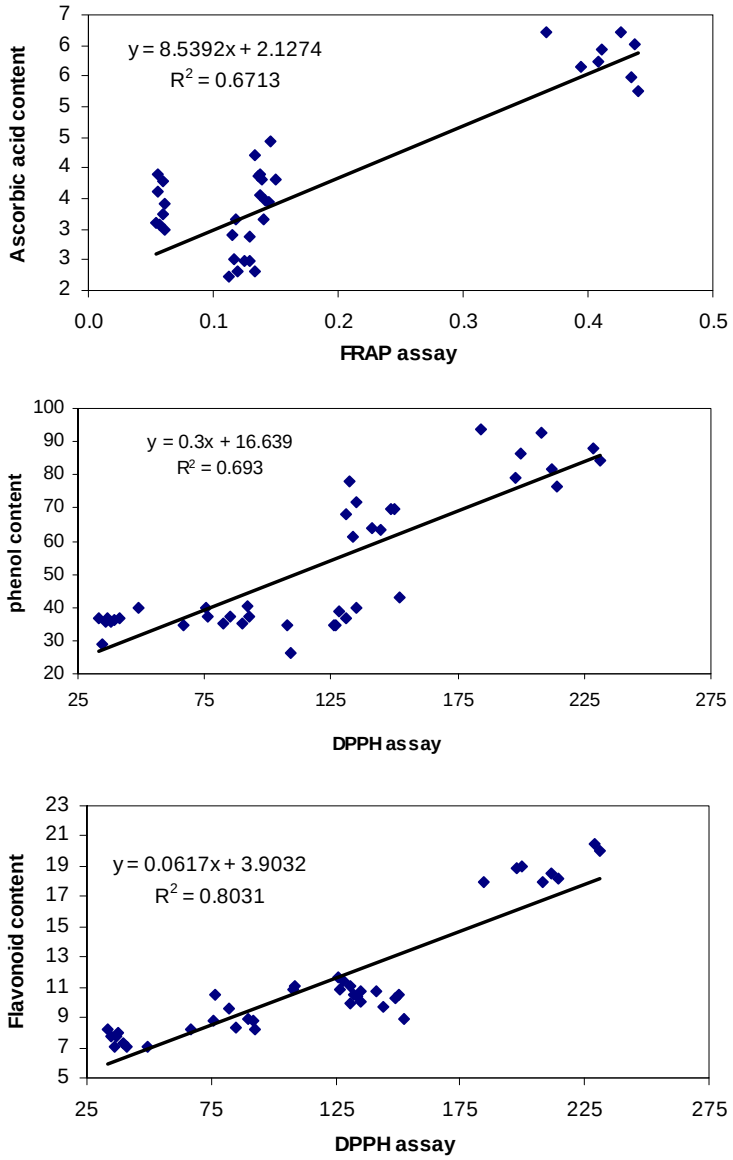

Figure 4. Correlation coefficients between antioxidant activity (FRAP and DPPH assay) and ascorbic acid (a), phenol (b) and flavonoid (c) contents.

Discussion

The mode of antioxidant action, which serves as a defensive system in all stages of plant development, depends on plant's growth, developmental stage and sample specificity (Howard et al., 1994; Deepa et al., 2007).

The significant variation in antioxidant properties, total phenols, flavonoid and ascorbic acid content between different stages of *T. transcaspicus* growth indicates that the potential efficacy of antioxidants in this plant varies considerably during maturity stages. The FRAP assay allows the overall estimation of water-soluble compounds influencing the total antioxidant activity (Deepa et al., 2007). By using this assay, our results showed an enhanced ascorbic acid level (as a compound water-soluble, R^2 = 0.67) during different stages, and also increased ferric reducing antioxidant activity of the extracts. Furthermore, we showed that the highest antioxidant activity was obtained in period 5, in which the ascorbic acid content was also the highest. In relation to our data, Proteggente et al. assessed the antioxidant activity of extracts from regularly consumed fruit and vegetables using FRAP assay, and found that the indices were well correlated with vitamin C content. The BCB assay showed that during all stages of growth, all samples were capable of inhibiting lipid peroxidation (Proteggenete et al., 2002). Current studies have been focused on a proposed role of carotenoids as lipid antioxidants, which can protect plants against destructive processes mediated by singlet oxygens and free radicals (Menichini et al., 2009). Although

the value of compounds was not assessed in this study, reports indicated that carotenoid concentration increased in the oldest leaves of acerola genotypes (Lima et al., 2005). Therefore, it can be concluded that the level of carotenoid concentration in step 5 might be responsible for enhanced antioxidant activity in the BCB assay.

In agreement with Pyo et al. (2004), our results suggested a linear correlation between DPPH radical scavenging activity and phenol and flavonoid concentrations (Figures 1b and 1c). These relationships could be due to the fact that both DPPH and the Folin–Ciocalteu methods are based on redox balances in phenols (Huang et al., 2005). The radical scavenging activity of phenolic compounds mostly depends on their molecular structure, due to the availability of phenolic hydrogens and stabilization of the resulting phenoxyl radicals formed by hydrogen donation. The changes in flavonoid composition during maturation most likely affected the antioxidant activity. It has been reported that some flavonoids such as patuletin derivatives show significant activity against DPPH radical and enhanced radical scavenging capacity, whereas in spinacetin derivatives radical scavenging capacity is reduced. Furthermore, it has been indicated that flavonoids with no, minor or high activity against free radicals varied in concentration in response to maturation (Pandjaitan et al., 2005). Therefore, the connection between the particular antioxidant agents and antioxidant activity is difficult to explain on the basis of quantitative analysis (Capecka et al., 2005). The synergism between antioxidants in mixture makes the antioxidant activity not only depend on the concentration, but also on the structure and the interaction between the antioxidants (Conforti et al., 2007). Therefore, the antioxidant activity did not always exhibit an additive or synergistic effect equal to the antioxidant content individually (Howard et al., 2000). Our results revealed that anti-oxidant behavior, as radical scavenger, oxidation inhibitor and reducing agent, differs in 5 growth stages of *T. transcaspicus*. This discrepancy may be due to different mechanisms involved in these stages of growth. Understanding the biochemical changes in plant physiology, food science, nutrition and health should stimulate interest in maximizing beneficial effects of plants in diet.

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Cytogenetic study and pollen viability of four populations of *Trigonella spruneriana* Boiss. (Fabaceae) in Iran

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Abstract

In the present paper, the cytogenetic study including meiotic chromosome number and behavior along with pollen viability were performed in 4 populations of *Trigonella spruneriana* Boiss. This is the first cytogenetic report of the taxon. All populations are diploid and possess $2n = 2x = 16$ chromosome number, which is consistent with the proposed basic number of $x = 8$. In addition, some meiotic irregularities observed in different populations included chromosomes stickiness, B-chromosomes, chromosome bridges resulting from stickiness, the occurrence of laggard chromosomes, formation of micronuclei in tetrad cells and cytomixis. The highest and the lowest percentages of pollen viability were observed in populations SPR 658 and SPR 566, respectively.

***Keywords*:** chromosome number, Iran, meiotic behavior, pollen viability, *Trigonella spruneriana*

Introduction

The genus *Trigonella* L. is a member of the tribe Trifolieae of family Fabaceae, the second largest family of flowering plants in the world with 650 genera and 18000 species (Dangi et al., 2004). In Flora Iranica (Rechinger, 1984) the genus is represented by 58 annual and perennial species in 12 categories. *T. spruneriana* belongs to *T.* sect. *Cylindricae*. This category is represented with 3 annual members in Iran.

Several cytological investigations have been conducted in *Trigonella* (Singh and Roy, 1970; Singh and Singh, 1976; Agarwal and Gupta, 1983; Ahmad et al., 1999; Dundas et al., 2006; Aykut et al., 2009; Martin et al., 2008, 2011a, 2011b; Ranjbar et al., 2011). The number of mitotic chromosome was first reported by Kamari and Papatsou (1973) on an accession from Nisyros (Aegean Sea) of *T. balansae* Boiss. and Reuter, an annual pasture legume of Eurasian origin. Martin et al. (2010) analyzed karyotype of *T. spruneriana* and 9 species of the *T.* sect. *Cylindricae* in Turkey. There is only a few information about karyotype and phylogenetic relationships as well as meiotic behavior in the genus due to limited studies.

Basic information on the meiotic behavior and estimation of pollen viability are useful for germplasm characterization and identification of genetic variability, biodiversity, and evolutionary processes of the species (Palm-Ailva et al., 2004). Meiosis is a process which is controlled by genes (Gottschalk and Kaul, 1974; Golubovskaya, 1979). Although the meiocyte is a highly specialized cell capable of producing four haploid cells, mutations, hybridizations, environmental stress, endogamy and other factors may alter the constitution or the expression of genes that act during meiosis (Utsunomiya et al., 2002). The amount and quality of pollen produced by a flower is an important component of fitness. Pollen quality is often represented to pollen viability, i.e., the proportion of pollen grains that are viable (Stanley and Linskens, 1974; Heslop-Harrison et al., 1984). Because of its importance in fertilization and therefore production, several studies have focused on investigation of pollen viability (Asma, 2008).

Materials and Methods

Cytogenetics

The chromosome number and meiotic behavior were analyzed in 4 populations of *T. spruneriana* which were collected from different regions within the natural geographical distribution of *T. spruneriana* during several excursions in Iran (table 1). Fifteen flower buds at an appropriate stage of development were fixed in 96% ethanol, chloroform and propionic acid (6:3:2) for 24 h at room temperature and then stored in 70% ethanol at

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4 °C until used. Anthers were squashed and stained with 2% acetocarmine. All slides were made permanent by the Venetian turpentine. Photographs of chromosomes were taken on an Olympus BX-51 photomicroscope at initial magnification of X 1000. The chromosome number was counted from well-spread metaphases in intact cells, by direct observation and from photomicrographs.

Pollen viability

Pollen stainability was considered as an indication of pollen viability. For this purpose pollen grains were first obtained from flowers of herbarium specimen and then stained with acetocarmin/glycerin (1:1). Slides were stored at room temperature for 24-48 hours. The stainability was determined using samples of 1000 pollen grains per flower. Slides were examined and documented with an Olympus BX-51 photomicroscope.

Table 1. Studied populations of *T. spruneriana* and their acronyms.

Taxa	Herbarium number	Altitude (m)	Location	Date	Collector name	Abbreviation
T. spruneriana	BASU 25667	1405	Azerbaijan Gharbi: Movana toward Salmas, 40 km to Salmas (37°.85'N, 44°.75' E)	19.5.2011	Ranjbar & Hajmoradi	SPR 667
T. spruneriana	BASU 23658	1100	Lorestan: Lorestan University (33°.50'N, 48°.45' E)	22.4.2009	Ranjbar & Hajmoradi	SPR 658
T. spruneriana	BASU 23689	1450	Lorestan: Dowrud, toward lake (33°.55'N, 49°E)	22.4.2009	Ranjbar & Hajmoradi	SPR 689
T. spruneriana	BASU 23566	1800	Kohgiluyeh and Boyer-Ahmad: Lordegan to Yasuj, 30 km before Pataveh (31°.75'N, 51°.25'E)	6.4.2010	Ranjbar & Hajmoradi	SPR 566

Results

Cytogenetics

With respect to meiotic chromosome number, meiotic stages, as well as abnormalities observed in each stage and data are presented in table 2. All populations are diploid and possess a chromosome number of $2n = 2x = 16$, which is consistent with the proposed basic number of $x = 8$. A total of 468 diakinesis/metaphases I (D/MI), 432 anaphase I/telophase I (AI/TI), 312 metaphase II (MII), and 1031 anaphase II/telophase II (AII/MII) cells were analyzed. Chromosomes stickiness, B-chromosomes, chromosome bridges resulting from stickiness, the occurrence of laggard chromosomes, formation of micronuclei in tetrad cells and cytomixis were meiotic irregularities, which observed in the above populations of *T. spruneriana.*

Pollen viability

The results of comparison between meiotic behaviour and pollen viability showed the highest (99.9) and lowest (16.2) percentages of the stained pollens in populations SPR 658 and SPR 566, respectively. This result indicates that irregularities observed at meiosis probably have a direct relation with species fertility. The pollen viability of examined species are described in table 2 and illustrated in figure 2.

Discussion

Meiosis is highly coherent and the process is genetically programmed which comprises of pairing homologous chromosomes, crossing over, reduced in chromosome number, and lacking of S period between the two divisions. Similar to any other biological process, all the sequential steps involved in meiosis are controlled by a large array

of genes (Ramana, 1974; Mok and Peloquin, 1975; Mok et al., 1976; Koduru and Rao, 1981; Falistocco et al., 1994). Mutation in any of these genes that govern micro or megasporogenesis from pre-meiotic to post meiotic events can lead to serious anomalies in the whole process resulting in the genetically aberrant end products having adverse impact on fertility and overall reproductive efficiency of the species (Lattoo et al., 2006). Furthermore, many abnormalities affecting plant fertility or causing total male sterility have been detected during the evaluation of meiotic behavior in some species.

Chromosome stickiness may be caused by genetic and environmental factors, and several agents have been reported to cause chromosome stickiness (Pagliarini, 2000). Sticky chromosomes along with laggards were found in different stages such as D/MI (figures 1B and F) and MII (figures 1M and T). Chromosome bridges resulting from stickiness were observed in populations of SPR 689 and SPR 566 (figures 1H and K). The thickness of bridges and number of chromosomes involved in their formation are varied among different meiocytes. Genetic as well as environmental factors have been considered as the reason for chromosome stickiness in different plant species (Nirmala and Rao, 1996). The phenomenon of cytomixis consists of the migration of chromosome between meiocytes through cytoplasmic connection. Since cytomixis creates variation in the chromosome number of the gametes, it can be considered as an important mechanism of evolution (Ghaffari, 2006). The highest degree of cytomixis occurred in population of SPR 566 (figures 1O and P). There was another abnormality (asynchronous nucleus) in metaphase II (figure 1N) that was found in all studied populations except SPR 658 population.

Table2. Characterization of meiotic behaviour of pollen mother cells (PMCs) in 4 populations of *T. spruneriana*.

Meiotic characters	**SPR 566**	**SPR 667**	**SPR 689**	**SPR 658**
Cell number	449	631	636	244
D/MI	186	118	125	39
% D/MI	41.42	18.70	19.65	15.98
% Cytomixis	2.15	5.93	0	0
% B-chromosome	0	0.84	0	0
% Sticky and laggard chromosome	3.22	11.86	13.87	5.1
AI/TI	166	141	71	54
% AI/TI	36.97	22.34	11.16	22.13
% Sticky and laggard chromosome	0.6	0	4.21	0
% Bridge	0.6	0	1.4	0
% Micronucleus	0	2.12	0	0
% Cytomixis	0	0	1.4	0
MII	97	75	97	43
% MII	21.6	11.88	15.25	17.62
% Sticky and laggard chromosome	1.03	12	1.03	0
% Asynchronous nucleus	2.06	6.66	4.1	0
% Cytomixis	24.74	5.33	0	0
AII/TII	265	297	343	108
% AII/TII	59.02	47.06	53.93	44.26
% Micronucleus	0	0.33	0	0
% Cytomixis	7.79	0	0.2	4.32
% Asynchronous nucleus	0	0	0	0.92
% Pollen viability	16.2	94	96.5	99.9

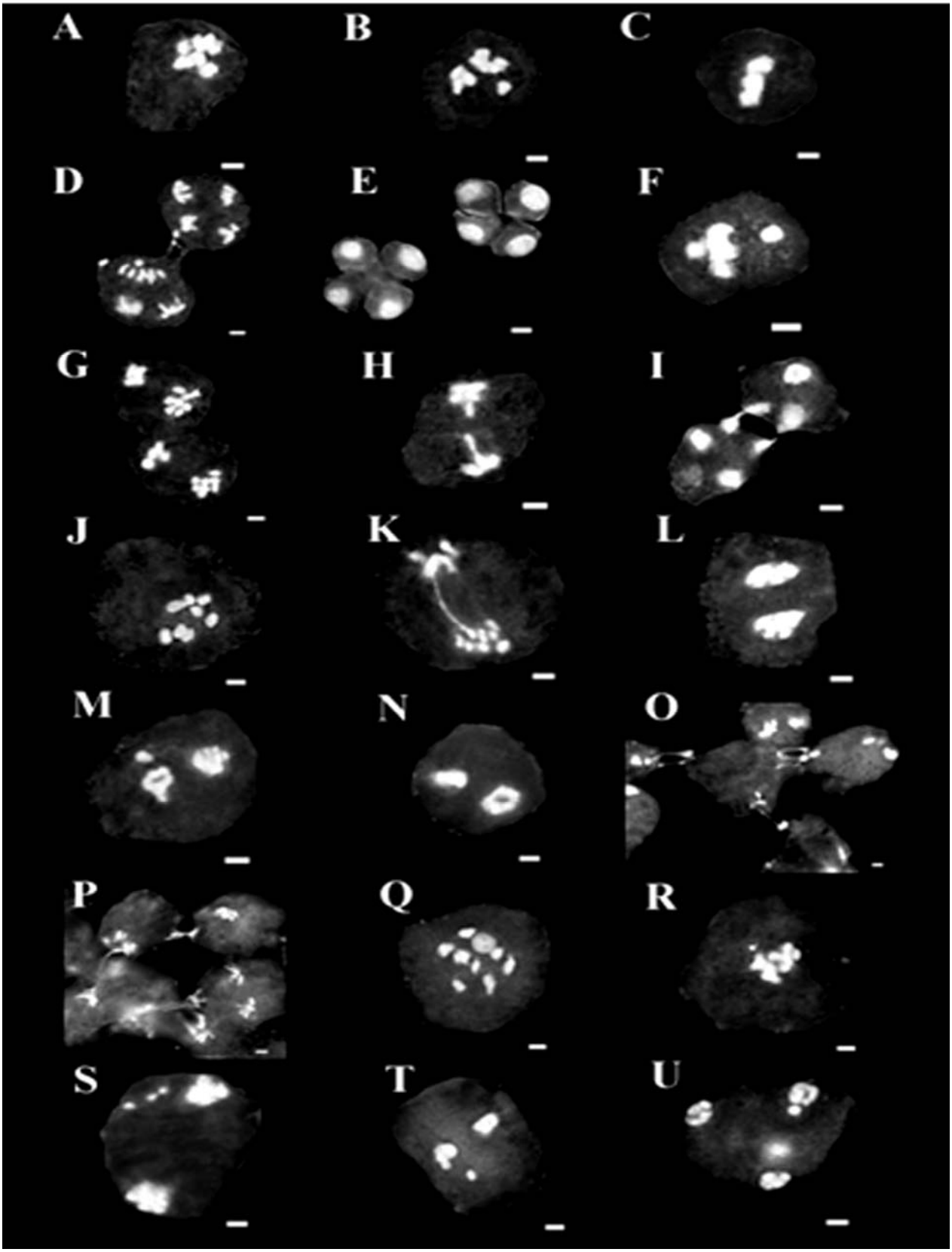

Figure 1. (A – E) Representative meiotic cells of population SPR 658. (A) Diakinesis, (B**)** Sticky chromosomes, (C) Metaphase I, (D) Cytomixis, (E) Tetrad, (F – I) Representative meiotic cells of population SPR 689, (F) Laggard chromosome, (G) Anaphase I, (H) Bridge, (I) Cytomixis, (J – P) Representative meiotic cells of population SPR 566, (J) Diakinesis, (K) Bridge, (L) Telophase I, (M) Laggard chromosome, (N) Asynchronous nucleus, (O) Cytomixis in metaphase II, (P) Cytomixis in anaphase II, (Q – U) Representative meiotic cells of population SPR 667, (Q) Diplotene, (R) B-chromosome, (S) Micronucleus, (T) Laggard chromosome, (U) Micronucleus. Scale bar = 10 μm.

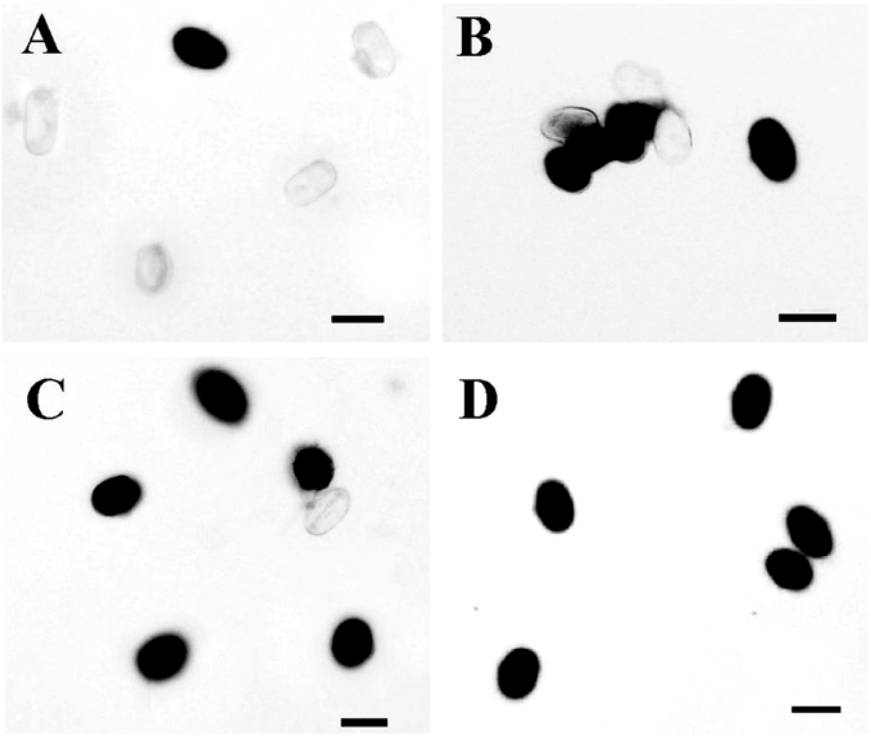

Figure 2. Pollen viability in different populations of *T. spruneriana*. (A) SPR 566, (B) SPR 667, (C) SPR 689, (D) SPR 658. Scale bars: 25 µm.

The highest degree of asynchronous nucleus was observed in population of SPR 667 (%6.6). B-chromosomes or extra chromosomes that occur in addition to the standard or A-chromosomes in some of the plants, are smaller than other chromosomes and do not form any synapsis with them. A large number of B-chromosomes have shown a negative effect on the growth and vigor of the plants, whereas in low numbers they may have some benefits for plant (Jones and Houben, 2003). B-chromosomes were only observed in population of SPR 667 (figure 1R). Chromosomes that produced micronuclei during meiosis were eliminated from microspores as microcytes. The micronucleus reached the microspore wall and formed a kind of bud, separated from the microspore. The eliminated microcytes gave origin to small and sterile pollen grains (Baptists-Giacomoelli et al., 2000). Micronucleus was only seen in population of SPR 667 (figures 1S and U). The highest and lowest abnormalities were seen in populations of SPR 566 and SPR 658, respectively.

The high percentage of stained pollen grains (≥94%) was recorded for 3 populations of *T. spruneriana*. This result is predictable based on meiotic behavior data and of the low percentages of irregularities in these populations (table 3). So in populations that chromatin is translocated in their PMCs either does not occur or occurs at a very low frequency, meiotic abnormalities are almost negligible and most of the pollen grains are fertile.

In contrast, a low percentage of pollen viability (16.2%) in population of SPR 566 can be explained by having high percent of cytomixis. In this population a relatively high frequency of chromatin transfers in different stages of meiosis and consequently, low pollen viability (16.2%) was observed. So, it can be concluded that cytomixis affects the meiotic course considerably and results in reduced pollen viability. Lattoo et al. (2006) and Singhal and Kumar (2008) also showed that there is a direct relationship between occurrence of cytomixis and reduced pollen viability.

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Blastema cells derived from rabbit ear show stem cell characteristics

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Abstract

Regeneration is a biological phenomenon, which takes place via two main mechanisms: first, dedifferentiation of mature cells followed by their differentiation into functional new cells and second, activation of endogenous somatic stem cells for regeneration of damaged or lost tissues. One of the best examples of healing process in mammals is the regeneration of damaged pinna in rabbits by blastema tissue. The aim of present study was to investigate culture requirements, proliferative properties and expression of some stemness factors in cells derived from regenerating blastema tissue obtained from rabbit pinna *in vitro*. The regenerating tissues were obtained from male New Zealand white rabbits by double punching of the pinna and cell culture conditions were set to derive and enrich the self renewing cells for further characterisation. The cells were subjected to survival and growth examinations *in vitro*, and expression of several stemness factors was studied in these cells using reverse transcription polymerase chain reaction (RT-PCR). Results revealed that the derived cells are rather immortal, as they have been growing for more than 120 passages in culture up until this report. Furthermore, RT-PCR and flow cytometry analyses showed that these cells express a number of stemness related genes including *Oct4* and *Sox2*. In conclusion, in this study, stem like cells were derived from blastema tissue of rabbit ears for the first time, showing great self renewing capacity, which provides a suitable *in vitro* model for regeneration studies. Moreover, they could be considered as a good source of stem like cells for future applications.

***Keywords*:** regeneration, blastema tissue, pluripotency, stem cell

Introduction

Derivation and applications of stem cells in recent years has taken the ground in many directions, including human health, as one of the most potential emerging fields. Since first radical report of successful derivation of human embryonic stem cells (ESCs) (Thomson et al., 1998), intense efforts have been dedicated to derive cells suitable for clinical applications. Due to several drawbacks, including ethical concerns and high risk of tumour development, ESCs are considered as low potential for therapiutic purposes (Stocum, 2002).

Meanwhile, several alternatives are being considered in parallel to cover the high expectations in regenerative medicine from stem cells. Among these, induction of dedifferentiation in somatic cells, and derivation and amplification of endogenous adult stem cells are of special interest for their potentials in regenerative medicine.

Several methods have been introduced for induction of dedifferentiation in differentiated cells to reach the cells with stemness features, including somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997), induction of reprogramming in somatic cells by the fusion of cells with ES cells (Do and Scholer, 2004), and treatment of differentiated cells with extracts of pluripotent cells (Freberg et al., 2007; Hansis et al., 2004; Taranger et al., 2005). However, these methods proved to be too inefficient to be used in therapeutical programs. In 2006, direct reprogramming of somatic cells towards pluripotent state, by using defined transcription factors, was reported by Takahashi and Yamanaka (Takahashi and Yamanaka, 2006). In this approach, they transduced mouse embryonic fibroblasts (MEFs) with four transcription factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*). One of the main drawbacks of induced Pluripotent Stem (iPS) cell

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technology is employment of exogenous genetic factors (viral based constructs) for induction of reprogramming. In addition, some of these reprogramming factors (c-Myc and Klf4) have an oncogenic nature.

Regeneration is usually defined as reformation of body parts that are lost by injury. This phenomenon can occur in various styles, including production of dense collagen scar or through a scar free approach which is called epimorphic regeneration (Stocum, 2006). In invertebrate species, regeneration of lost parts is widely observed, while most vertebrates do not have such a remarkable ability (Brockes and Kumar, 2005; Endo et al., 2007; Masaki and Ide, 2007; Mochii et al., 2007). Among vertebrate species, urodeles and teleosts retain high ability for regeneration and are able to replace lost appendages (Poss et al., 2003).

Nearly all animals possess the capability of regeneration, but relatively only few species have ability of epimorphic regeneration. Our current knowledge about this phenomenon is largely gained from studies on lower vertebrates, particularly on amphibians (Brockes and Kumar, 2002), Nevertheless, some few mammals have also shown this ability. Rabbit ears are known to be capable of regenerating tissues producing by punching their pinnas (Dyson and Joseph, 1971; Goss and Grimes, 1972; Grimes and Goss, 1970). All other mammalian ears are incapable of regenerating full thickness. Instead, their inner and outer epidermis simply heal arround the margins and scar is formed, whereas, in the rabbits a circular blastema tissue is developed. Studies have demonstrated that regenerating rabbit ear tissues and cells have fascinating properties (Mahdavi-Shahri et al., 2008).

The ultimate goal of regenerating studies is to understand how lost or damaged cells are replaced and which mechanisms are involved in this phenomenon. Various mechanisms have been observed to be associated with regeneration in animals including, dedifferentiation, transdifferentiation and activation of somatic stem cells. For example, in zebrafish dedifferentiation and proliferation of cadiomyocytes has been reported to be involved in regeneration of the missing tissue of heart (Jopling et al., 2010; Kikuchi et al., 2010), or transdifferentiation was first observed in the regenerating lens of newts (Wolff, 1895), and activation of somatic stem cells located in a niche has been reported as the mechanism involved in regeneration (as occurs with blood) (Jopling et al., 2011).

Since one of the mechanisms associated with regeneration is dedifferentiation, studying this phenomenon in some animals can be useful as a simple system for decoding and promoting reprogramming technology.

In present study, stem like cells (SLCs) were derived from rabbit pinnas for the first time, and their morphology, growth rate and viability were investigated *in vitro*. Also, expression of genes such as *Oct4* and *Sox2*, which are characteristics of pluripotent cells was profiled.

Materials and Methods

Animals

3-6 month old male New Zealand white rabbits (*Oryctolagus cuniculus*), were purchased from Razi Vaccine and Serum Research Institute (RVSRI) (Mashhad, Iran). Rabbits were kept under standard conditions, fed with standard rabbit chow (Javaneh Khorassan, Iran) and tap water.

Preparing and culture of regenerating tissues

Rabbit pinnas were shaved, cleaned with 70% ethanol and punched to make holes (2 mm in diameter). Punches were made in areas between the medial ear artery and the marginal ear veins, where there are few major vessels. Two days after first punching, the second punches (4 mm in diameter) were made and O-shaped rings were obtained. The rings were washed with physiological serum and culture medium for several times, and transferred into the six-well plates for culture.

Culture and collection of outgrowth cells from the regenerating tissues

Some cells from the rings started to grow and attach to the surface of the plates. These cells were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Scotland) supplemented with 15% fetal bovine serum (FBS; Gibco, Scotland) and 1% penicillin and streptomycin (Biosera, UK), and incubated at 37°C in a humidified atmosphere containing 5% CO_2. About one week later, adherent cells which were confluent, were detached using 0.25% trypsin-EDTA (Gibco, Scotland) and transferred into 25-cm^2 culture flasks. The culture medium was refreshed every 2 days and cells were passaged twice a week. These cells were designated as SLCs. The cells were frozen in liquid nitrogen using freezing solution, containing 10% dimethyl sulfoxide (DMSO, Merck, Germany) and 90% FBS (Gibco, Scotland).

Karyotyping analysis

In order to karyotype the cells, they were first

treated with vinblastine solution (0.5 µg/ml) (Sobhan Oncology Company, Iran) for 4 h and then detached from the flasks using trypsin-EDTA. Then KCl (0.56 g /100 ml ddH_2O) (Merck, Germany) was added to the cells and cold acetic acid/methanol solution (Merck, Germany) (3:1) was used to fix them. The cells were finally stained with 20% Giemsa solution (Merck, Germany) and analysed by light microscopy (Olympus, Japan).

RNA extraction and RT-PCR analysis

Total RNA was extracted from the SLCs at passage 5 using RNX-Plus solution (CinnaGen, Iran), according to the manufacturer's guidelines. The RNAs were then treated with DNaseI (5Prime, Germany), and their concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA). 1 µg of the total RNA was used for reverse transcription in a 20 µl mixture containing 1 µl of Oligo(dT) primer (10 pmol) (Promega, USA), 2 µl dNTPs mix (10 mM) (CinnaGen, Iran), 4 µl cDNA synthesis buffer, 1 µl RNase inhibitor (40 u/µl) (Fermentas, Germany) and 1 µl M-MuLV reverse transcriptase (200 u/µl) (Fermentas, Germany) and DEPC-treated water (CinnaGen, Iran) to 20 µl. The reverse transcription was conducted at 42˚C for 1 h followed by 10 min incubation at 72˚C and samples were stored at -20˚C until use. The test cDNAs were normalised with controls using β-actin primers (Matin et al., 2004), and equal amount of each cDNA was used as template for PCR amplification with specific primer pairs, derived from the conserved regions of the reported sequences of human, rabbit and rat genes (*Oct4* and *Sox2*). The forward and reverse primer sequences are shown in table 1. Finally, 8 µl of PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and visualised under UV light. The expression levels were compared to mRNA levels of desired genes in NTERA2 cells (a generous gift from Professor Andrews, University of Sheffield) and human adipose tissue derived mesenchymal stem cells (AT-MSCs), which were derived in the lab as described elsewhere (Ahmadian Kia et al., 2011).

Flow cytometry analysis

For flow cytometry analysis, cells were detached after treatment with trypsin-EDTA for 5 min at 37°C, washed with phosphate buffered saline (PBS) containing 5% FBS for three times, and fixed with 4% paraformaldehyde/PBS (Sigma, Germany) for 20 min at 4°C. After three times washing, the cells were permeablised with 0.2% digitonin (Sigma, Germany) for 10 min at 4°C and stained with Oct4 primary antibody (1:50) (Santa Cruz, USA) for 45 min at 4°C. The cells were then washed three times and incubated with FITC-conjugated anti-mouse IgG for another 30 min at 4°C. After final wash, cells were subjected to flow cytometry analysis using FACS Calibur (BD, USA) machine. Also, cells incubated with FITC-conjugated secondary antibody, in the absence of primary antibody were used as negative control. HFF3 cells (a generous gift from Royan Institute, Tehran, Iran) were also used as a control in this experiment.

Results

Derivation and culture of stem like cells (SLCs) from rabbit pinna

The punched O-shaped rings from male New Zealand rabbit pinnas (figure 1) were able to produce outgrowth cells after 7-10 days in culture. The cells had a spindle like morphology, similar to Mesenchymal Stem Cells (MSCs) (figure 2). The growth curve of cells at passage 5 was analysed for 6 days in the media containing different percentages of FBS (figure 3). SLCs could grow better in DMEM medium supplemented with 15% FBS. The cells were grown for over a year (more than 120 passages) in the lab without obvious changes in their growth rate and morphology. They were also subjected to karyotyping analysis at passage 65, where no detectable abnormality was observed (figure 4).

Stemness factors are expressed in SLCs derived from rabbit pinna

The very well known stem cell molecular markers of Oct4 and Sox2 were shown to be expressed in these cells at mRNA level by RT-PCR. Meanwhile, the overall level of expression for *Oct4* was lower than that in the pluripotent NTERA2 cells and multipotent human adipose derived mesenchymal stem cells (figure 5). Although the level of *Sox2* mRNA expression was lower than that in the NTERA2 cells, but it seemed to be higher than its expression in AT-MSCs (figure 5). Expression of *Oct4* was further verified at protein level. Flow cytometry analysis revealed that 20% of the cells were Oct4 positive (figure 6A) which was far more than the human fibroblast cells (HFF3) which were used as control (figure 6B).

Table 1. Primer sequences used in RT-PCR experiments.

Gene Name	Forward primer	Reverse primer	Amplicon (bps)	No. of Cycles	Annealing temp. (˚C)
β-actin	5'- ATCTGGCACCACACCTTCTA CAATGAGCTGCG- 3'	5'-CGTCATACTCCTGCTTGCTG ATCCACATCTGC-3'	838	28	62
Oct4	5'- GAACATGTGTAAGCTGCGGCC-3'	5'- CCCTTCTGGCGCCGGTTAC -3'	270	40	58
Sox2	5'- AGCATGATGCAGGACCAG-3'	5'- GGAGTGGGAGGAAGAGGT-3'	269	40	52

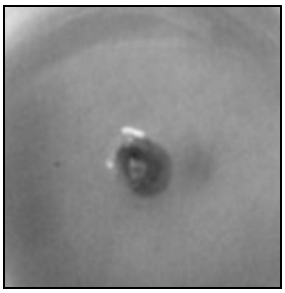

Figure 1. The O-shaped rings as punched from rabbit pinna in culture medium.

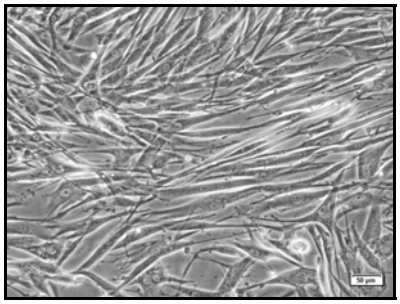

Figure 2. Morphology of SLCs in fifth passage (scale bar: 50 µm).

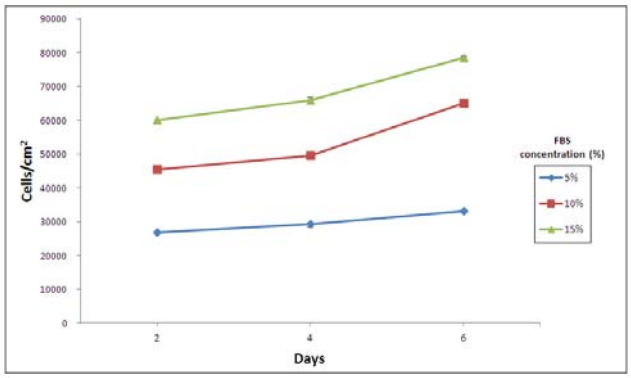

Figure 3. Growth rate analysis of SLCs grown in media containing various percentages of FBS, as examined by counting the cells at different time points (data are shown as Mean+/-SD).

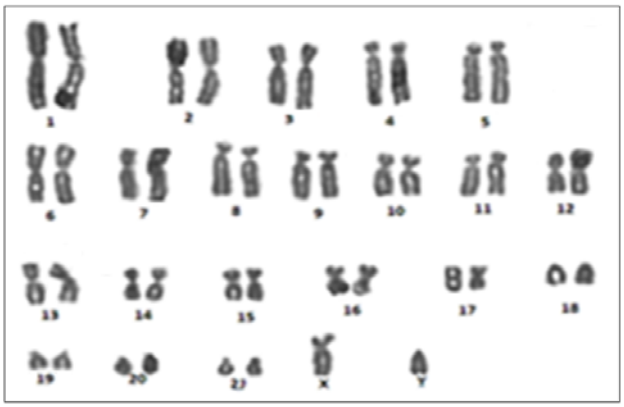

Figure 4. Karyotype analysis of SLCs, derived from male New Zealand white rabbit pinna at passages 65.

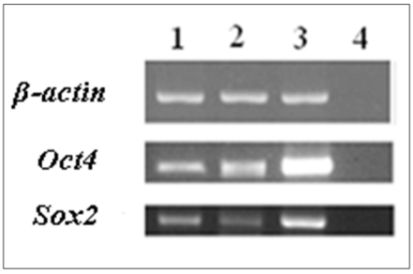

Figure 5. Semi-quantitative RT-PCR analysis of mRNA level for *β-actin*, *Oct4*, and *Sox2* genes. 1: SLCs 2: rat MSCs, 3: NTERA-2 cells, 4: non-template negative control.

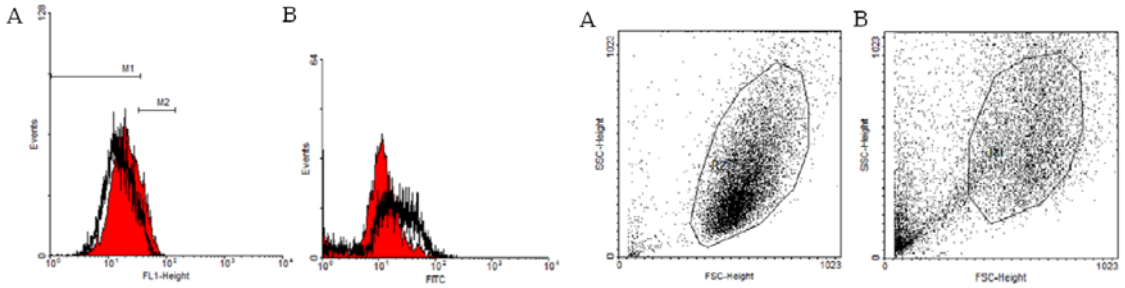

Figure 6. Dot plots (upper panel) and histograms (lower panel) of flow cytometry analysis for Oct4 protein expression in SLCs (A), and human fibroblast cells (HFF3) (B).

Discussion

In biology, regeneration is a process in which a lost or damaged part of the body could heal so that the original function is restored. Some vertebrates like newts can regenerate injured limbs in two major steps, first dedifferentiation of the adult cells to a state similar to embryonic cells and second, development of fresh tissues from these cells (Odelberg, 2004). Simpler animals, like planarian have been shown to be far more efficient in regeneration by retaining clusters of stem cells within their bodies that migrate to the damaged parts and differentiate to provide the required missing tissues (Agata and Umesono, 2008). Beside these famous examples, tissue regeneration, although in lower extent, has also been reported in vertebrates including, salamander, zebrafish and few mammals like deer antler, mouse fingertip, MRL mouse and rabbits. A well known model for epimorphic regeneration in mammals is rabbit ear tissue regeneration, where all of the removed tissues are healed without any scar. This phenomenon was at first discovered by Markelova in 1953 and has been reviewed regularly since then (Joseph and Dyson, 1966; Goss and Grimes, 1972; Williams-Boyce and Daniel, 1980). This scarless regeneration was attributed to unique features of blastema tissue, with less-differentiated local cells, which proliferate and differentiate to new sheet of cartilage as it regenerates in a centripetal direction (Goss and Grimes, 1975). Since cells inside this tissue can establish all of the lost cells, we could use them in the field of regenerative medicine and also blastema tissue can be considered as a pool of powerful unique cells.

Having this interesting background, current study was aimed to culture these cells *in vitro* and determine their possible long term immortality in one hand and characterise their stemness state using the defined criteria based on stem cell markers expression on the other hand. These cells were successfully derived from the 2-day old ear rings. The number of passaging of these cells was phenomenal, reaching more than 120 passages until this report, without obvious changes in their morphology and growth rate. They kept these features even in the samples grown from the frozen cells. Expression of genes such as *Oct4* and *Sox2*, which are characteristic for ESCs, was detected in the derived SLCs.

In summary, to our best of knowledge this is the first time that immortal cells, sharing characteristics of pluripotent stem cells are derived from rabbit ears. These cells, which are designated as stem like cells (SLCs) in here, would serve as a good model for developmental biology studies as well as human disease preclinical tests. However, their characteristics need to be explored in more details.

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Identification of Safflower as a fraud in commercial Saffron using RAPD/SCAR marker

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Abstract

Saffron (*Crocus sativus*) is the most valuable and indigenous crop in Iran. The stigmas of flower are used as a popular natural flavouring, colouring and medicinal agent. However, the market suffers from frauds in this plant such as mixing with safflower petals due to high profit. Identification of these frauds with conventional and biochemical methods is difficult and low sensitive. Therefore, application of molecular markers such as random amplified polymorphic DNA (RAPD)/sequence characterized amplified regions (SCAR) is being considered as an alternative. In this study, DNA was extracted from dry stigmas of 5 Saffron accessions and dry petals of 7 safflower cultivars. RAPD reactions with ten 15-mer random primers resulted in two specific monomorphic bands (500 and 700 bp) for safflower, while they were absent in saffron accessions. PCR analysis with specific SCAR primers amplified two specific bands (414 and 589 bp) for safflowers in different combinations of saffron stigmas and safflower petals. This was the case with very low rates or 1% of safflower. Therefore, this method seems to be suitable for fraud identification of safflower petals in commercial saffron samples.

***Keywords*:** fraud identification, RAPD/SCAR, safflower, Saffron

Introduction

Saffron as the most expensive agricultural product of the world has a special importance among the exported products of Iran. More than 80% of saffron production worldwide belongs to Iran (Trade Promotion Organization of Iran, 2009). Saffron is produced from dried stigmas of *Crocus sativus*, and is considered as the most expensive spice in the world (Amir Ghasemi, 2001). Each flower has three stigmas which weighted 5 mg, and, 200000 flowers must be carefully picked one by one in order to produce 1 kg spice (Kafi et al., 2003). So, its high value has made saffron the object of frequent adulteration, and also being the object of intense chemical and biotechnological research (Fernandez, 2004). Under the prevention of Food Adulterant Act, an adulterant is any material which is employed for the purposes of adulteration. Mixing of similar materials such as beet, pomegranate fibers, red-dyed silk fibers, the flowers of other plants such as *Carthamus tinctorius* or safflower, *Calendula officinalis* or marigold, arnica and tinted grasses are the most fraudulent activities in saffron (Kafi et al., 2003). The limitation of saffron production and also its high price caused some efforts for its artificial production and frauds in which one of the most common ways is adding safflower petals to saffron stigmas (Ghasemi, 2001). There are different ways for determining adulteration in saffron such as chemical (Haghighi et al., 2007; Lage and Cantrell, 2009) and physical (Tsimidou and Tsatsaroni, 1993; Cuko et al., 2003; Zalacain et al., 2005) measurements but their sensitivity is usually low. Recently, progressing in DNA techniques makes it possible to identify any unwanted biological materials in plant products, especially in saffron (Pardo et al., 2003; Dnyaneshwar et al., 2006). PCR has a high potential for adulterant detection due to its simplicity, sensitivity, specificity as well as rapid processing time and low cost (Vidal et al., 2007; Mafra et al., 2008; Reid et al., 2006). The PCR-based methods used for adulterant detection and authentication include the amplification using species specific primers, DNA fingerprinting methods like RAPD (Williams et al., 1990), PCR with arbitrary primers (AP-PCR) (Welsh and McClelland, 1990), DNA amplification fingerprinting (DAF) (Caetano Anolles et al., 1991), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz et al., 1994), PCR-RFLP (restriction fragment length polymorphism) (Konieczny and Ausubel, 1993). Among these, RAPD marker uses

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small and random primers. In order to solve the non-repeatability problem of RAPD marker and specific band identification, RAPD/SCAR marker was developed (Dnyaneshwar et al., 2006). Today, SCAR markers, made by RAPD sequences, are successfully used for both the identification of some crops such as grape (Vidal et al., 2000), olive (Hernandez et al., 2001; Dovwri et al., 2006) and the identification of frauds in other plants such as *Embelia ribes* (one of the important plants used in Indian traditional medicine) (Devaiah and Venkatasubramanian, 2008), *Phyllanthus emblica* (Dnyaneshwar et al. 2006), the fibers of bamboo (mainly used in the pulp, paper and charcoal industries) (Das et al., 2004), olive oil (Dovwri et al., 2006), commercial Pelargonium (Lesur et al. 2001), Korean ginseng (Park et al., 2006), poplar commercial clones (Fossati et al., 2005) and *Encephalartos* (the second largest genus of the cycads) (Prakash and Van Staden, 2008) and also for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2006). Devaiah and Venkatasubramanian (2008) used RAPD-SCAR marker for fraud identification in *Embelia ribes* which is the traditional pharmaceutical plant in India. In their study, RAPD reaction resulted in specific band (906 bp) for this species which was used for designing SCAR primer to detect this species among frauds. There are some reports about fraud identification and quality control in saffron by biochemical methods (Tsimidou and Tsatsaroni, 1993; Cuko et al., 2003; Haghighi et al., 2007; Lage and Cantrell, 2009; Maggi et al., 2011) but there is not any report of application of RAPD-SCAR method for fraud identification in saffron, specially about adding safflower petals. Lozano et al. (1999) used an HPLC method for simultaneous detection, identification and quantification of the secondary metabolites in commercial saffron and some possible artificial colorants. Regarding the sensitivity of molecular marker and the importance of fraud identification in saffron, we used RAPD/SCAR marker for identification of safflower frauds in commercial saffron.

Materials and Methods

Plant materials

Fresh leaf tissues and dry stigmas of saffron *(C. sativus)* samples or accessions were used in this experiment. They were collected from five different regions of Iran (Ghaen, Gonabad, Barakuh Gonabad, Torbat Heidariieh, and Science and Technology Park). Fresh leaf tissues and dry petals of seven safflower *(C. tinctorius)* varieties (IL-111, 2819, 279, K.W.3, K.W.6, K.W.16, 295), were provided from gene bank collection of Agricultural Research Center of Khorasan province, Iran.

RNA extraction and RAPD-PCR

DNA was isolated from fresh and dry as well as commercial samples of saffron and safflower using Modified Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Saghai-Maroof et al, 1984). In brief, fresh leaf tissues (60-110 mg) or dried tissues (30-50 mg) were ground in liquid nitrogen. Freshly prepared extraction buffer (containing Tris-HCl 1M (pH=7.5), NaCl 2.5 M, EDTA 0.5 M (pH = 8), CTAB 2%, PVP 2%, and BME 1%) was added. The following steps were done according to the Modified CTAB procedure of Saghai-Maroof et al. (1984). PCR was done by ten 15-mer random primers (table 1) in a reaction mixture of 25 µl volume. Each reaction tube contained 50 ng DNA, 1 U Taq DNA polymerase enzyme, 200 µM of each dNTPs, 1x *Taq* DNA polymerase buffer, 4 mM $MgCl_2$ and 10 pmol of each primer. Amplifications were carried out in a DNA thermal cycler (Biometra) using following parameters: 94°C for 3 min; 35 cycles at 94°C for 1 min, annealing in 3 centigrade degrees below melting temperature for 1min, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were loaded on agarose gel (1.2%) electrophoresis in 0.5X TBE buffer.

Selecting of monomorphic bands and DNA fragment sequencing

Amplicons, which were monomorphic for all the safflower varieties but absent in five saffron samples were identified (figure 1). The putative markers amplified by the random primer RAP5, were excised from agarose gel with sterile gel slicer and purified using Clean Qiagen Gel Extraction kit. The A-tailed DNA was ligated into a TA-vector using Rapid DNA ligation kit (Roche, Germany). The ligated vector was introduced into competent *Escherichia coli* strain DH5α according to the protocol of transformation by calcium chloride. The transformed colonies were picked up from the LB medium with ampicillin as selective agent. Recombinant plasmids were isolated from each overnight grown colony with High Pure Plasmid Isolation kit (Fermentas, Germany). Confirmation of the clones was done by digesting the recombinant plasmid using *sacI* enzyme.

Recombinant plasmids were sequenced by automated sequencer in Macrogen Inc. of Korea. Based on the sequencing, some pairs of SCAR primers were designed. The SCAR primer pairs

were used for PCR amplifications of genomic DNA from the seven safflower varieties and five samples of saffron and also the DNA extracted from the mixed plant material of safflower in saffron with different combinations (1 %, 1.5 %, 2.5 %, 5 %, 7% and 10 %). PCR reaction was done according to the volumes and cycle program of RAPD reaction which mentioned before by chossing 58°C as annealing temperature. Homology searches were performed within GenBank's non redundant database using the BLAST program.

Results

Identification of RAPD marker for safflower

High molecular weight genomic DNA was isolated successfully from all the fresh and dried tissues. RAPD reactions resulted in some monomorphic bands in a few primers. Of them, RAP5 primer produced distinct and reproducible amplification profile for all the DNAs. Primer RAP5 consistently amplified two intense bands of 473 and 717 bp for all the safflower varieties, which were absent in the saffron samples (figure 1). These bands, named as SAF-L717 and SAF-L473, were selected as putative safflower specific markers.

The length of the SAF-L473 and SAF-L717 marker sequences were 473 and 717 bp, respectively. BLAST results revealed that the SAF-L473 sequence has 74% homology with mitochondrial ccb206 gene (for cytochrome C biogenesis protein with the accession number of AM183222.2) of *Helianthus annuus*. Our sequence was submitted to NCBI with the gene bank number of GU183488.1. The SAF-L717 sequence has no similarity with any sequence in NCBI database but shows certain levels of sequence-similarity with some plant nucleotide sequsences in EMBL database such as the yellow starthistle *Centaurea solstitialis* cDNA with the ID of EMBL-Bank: EH764694 (84% identity). The SAF-L717 sequence was submitted to the NCBI with the accession number of GU183487.1. There was however no similar sequences in safflower genome for these two sequences.

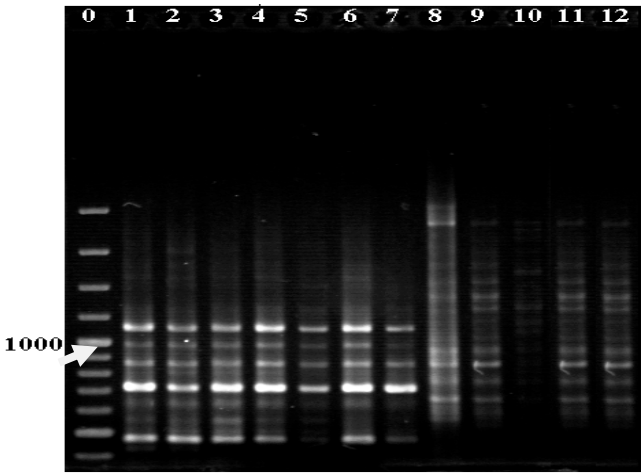

Figure 1. RAPD profiles of safflower varieties and saffron samples amplified with RAP5 on 1.2% agarose gel (0: Size marker (100 bp), lanes 1-7: Safflower varieties (1: IL-111, 2: 2819, 3: 279, 4:K.W.3, 5: K.W.6, 6: K.W.16, 7: 295), Lanes 8-12: Saffron samples; (8: Ghaen, 9: Gonabad, 10: Barakuh Gonabad, 11: Torbat Heidariieh, 12: Science and Technology Park)

Amplification Using SCAR Primers

Based on the sequencing, three pairs of SCAR oligonucleotide primers (SAF-L70 primer for SAF-L717 sequence, SAF-L40 and SAF-L4 for SAF-L473 sequence) (table 2) were designed by Primer3 online website which could amplify approximately 589, 414 and 412 bp of the genomic safflower DNA. The designed SCAR primer pairs were used to amplify genomic DNA from the 7 safflower varieties, while the DNA from the saffron specimens was not amplified by these primers. A single, distinct band of 414 bp was obtained from the DNA isolated from all the 7 safflower varieties and no non-specific amplification was observed in the 5 saffron samples in presence of SAF-L40 (figure 2). A single, distinct and brightly resolved band of 412 bp was obtained in DNA isolated from the safflower varieties and no non-specific amplification was observed in the saffron samples in presence of SAF-L4. Two distinct and brightly resolved bands of 589 and 300 bp were obtained in DNA isolated from the safflower varieties and no non-specific amplification was observed in the saffron samples in presence of SAF-L70 (figure 3).

Reduction of the annealing temperatures did not generate any fragment other than the SCAR bands, confirming the specificity of the SCAR primers for all the safflower varieties. The SCAR primers were used to distinguish safflower frauds in commercial saffron, even in low level of 1%, of safflower in saffron (figure 4).

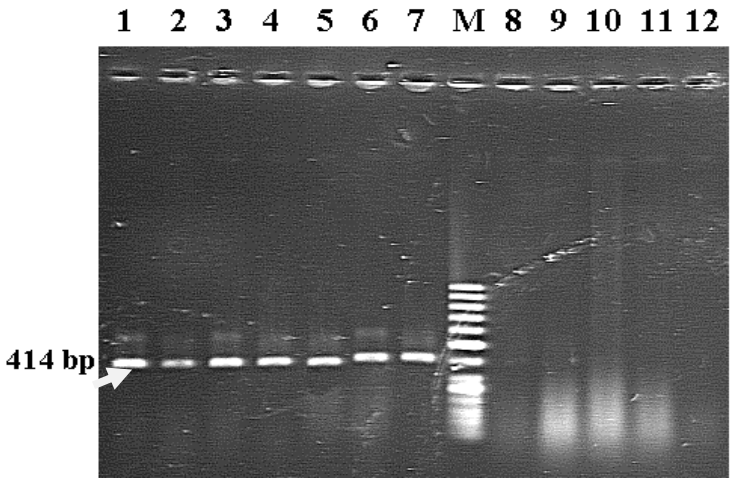

Figure 2. PCR amplification of safflower varieties using SAF-L40 on 1.2% agarose gel (M: Size marker (100 bp), lanes 1-7: safflower varieties (1: IL-111, 2: 2819, 3: 279, 4:K.W.3, 5: K.W.6, 6: K.W.16, 7: 295). Lanes 8-12: Saffron samples (8: Ghaen, 9: Gonabad, 10: Barakuh Gonabad, 11: Torbat Heidariieh, 12: Science and Technology Park).

Discussion

Identification of frauds and species is important for quality control of foods. There are examples of successful identification of species and even varieties in raw and processed materials such as textiles, seafood and plant products (Schubbert et al., 2008; Chapela et al., 2003). Molecular methods are suitable systems for tracing based on impurity in products, identification through DNA analysis. In fact DNA is unchanged and detectable in every cell, resistant to heat treatments and allows species identification (Perez and Garcia-Vazquez, 2004).

The samples which are used as fraud material are normally similar to the natural ones in morphology, and it makes their identification too difficult (Park et al., 2006). This problem can be solved by using chemical and molecular techniques specially the RAPD/SCAR method (Hernandez et al., 1999). In this study, we developed RAPD-SCAR marker for identification of safflower impurities (1%) in Saffron. In our RAPD analysis, non significant genetic polymorphism was observed among the safflower varieties and saffron samples. We selected two monomorphic bands SAF-L473 and SAF-L717 in safflower varieties for SCAR marker development. In SCAR, pairs of 20-25 bp specific primers can be used to amplify the characterized regions from genomic DNA under stringent conditions, which makes these markers more specific.

These results confirm the application of the designed primers as a qualitative diagnostic tool for identification of safflower impurities in saffron. However, for quantitative analysis of safflower content in the commercial saffron samples, advanced techniques such as real time PCR could be examined. Nevertheless, there is a pool of materials that can be used as adulterant for saffron. The adulterant may be phyllogenetically close or distinct from saffron and we are in process of developing primers for identification of such frequently used adulterants. In previous methods such as biochemical procedures, we had much impurities and it was also time consuming. So, this method enjoys from advantages of detection of impurities as low as 1% which is far from expectations in biochemical detection methods. This method is also used in frauds detection in some pharmaceutical plants such as *Phyllanthus emblica* and other economic plants such as *Bambusa* species, where the quality and quantity of paper pulp is greatly influenced by species (Das et al., 2004).

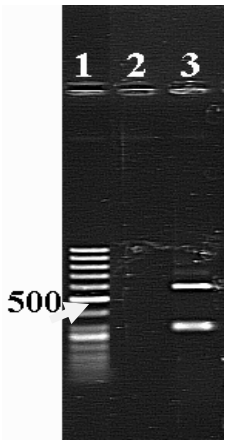

Figure 3. PCR amplification of safflower and saffron using SAF-L70 on 1.2% agarose gel (lane1: size marker (100 bp), lane2- PCR of saffron DNA (all of saffron samples) by SAF-70 primers, lane3: PCR with DNA mixture of safflower varieties by SAF-70 primers.

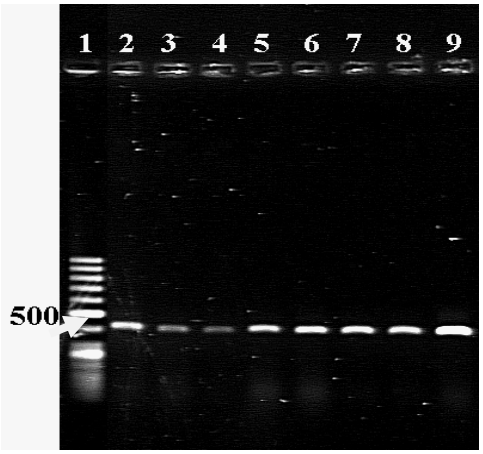

Figure 4. PCR amplification of genomic DNA extracted from mixed plant material of safflower in saffron using SAF-L4 on 1.2% agarose gel , (The numbers represent percentage of safflower in saffron samples as follows: 1: Size marker (50 bp), 2: 1% , lanes 3 and 4: 1.5%, lanes 5 and 6: 2.5%, 7: 5 % , 8:7%, 9: 10%).

Table1. The sequences of RAPD primers used in this experiment.

RAPD Primer	Primer sequence
RAP1	5′AACGACGAGCGTGAC 3′
RAP2	5′ GACAGCTTATCATCG 3′
RAP3	5′ ATGCAGGAGTCGCAT 3′
RAP4	5′AGTCATGCAACGCGC 3′
RAP5	5′GTATCACGAGGCCCT 3′
RAP6	5′GCTAGAGTAAGTAGT 3′
RAP7	5′ATGCGTCAGGCGTAG 3′
RAP8	5′TGCACTGCAGTGCAC 3′
RAP9	5′ GACTCCTGGATACCG 3′
RAP10	5′ GTAATACGACGGCCA 3′

Table 2. SCAR primers.

Primer	Sequence	Tm (ºC)
SAF-L 40 Forward	5′CCTCTCCTTTAACCCGAACAG 3′	58
SAF-L 40 Reverse	5′ATGGACTGAAGCTGGAATGAG 3′	
SAF-L 70 Forward	5′ TGAGCAGAGGAGGAGACTTG 3′	58
SAF-L 70 Reverse	5′GCCCTCAAGAAGAATACAGAGG 3′	
SAF-L 4 Forward	5′CCTCTCCTTTAACCCGAACAGCC 3′	60
SAF-L 4 Reverse	5′GGACTGAAGCTGGAATGAGAATAAC 3′	

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Maternal nicotine exposure-induced collagen pulmonary changes in Balb/C mice offspring's

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Abstract

Nicotine is an alkaloid by high level of addictive property that can quickly assimilate from smoker's lung. It passes from the placenta and gathers in the developing fetus. Our previous study showed that collagen type IV plays a critical role in basement membrane of different embryonic organs. In this study the effect of maternal nicotine was evaluated by collagen IV changes in lung of mice offspring during pre and postnatal period. Pregnant Balb/C mice were divided into 2 experimental and 2 control groups. Experimental group 1 received *3 mg/kg* nicotine intrapritoneally from day 5 of gestation to last day of pregnancy. Experimental group 2 received the same amount of nicotine during the same gestational days as well as 2 first week after birth. The control groups received the same volume of normal saline during the same periods. At the end of exposure times, all newborns were anesthetized and their lungs were removed and immunohistochemical study for tracing collagen was carried out. Our results showed that collagen reaction in the bronchial basement membrane (BBM) and extra cellular matrix (ECM) of the lung parenchyma experienced a remarkable increase when compared to the control ones. Cell necrosis definition in lung parenchyma of the experimental group 2 was the other finding that our investigation revealed. These data indicate that maternal nicotine exposure may induce a noticeable collagen increase with a reasonable amount in BBM and ECM of respiratory system of next generation.

***Keywords*:** respiratory system, nicotine, collagen IV, mouse

Introduction

Nicotine is an alkaloid obtainable from tobacco plant. It is one of the most important components of cigarette by high level of addictive property (Martin, 1970). Nicotine is a lucid liquid with an unpleasant odor that, when exposed to air, changes to brown (Catassi et al., 2008). Some of studies indicate that nicotine passes quickly from placenta and gets accumulated in the fetus and causes adverse effects on fetus development (Sung-HwaSohn et al., 2008; Harmanjatinder et al., 2002; Taylor and Wadsworth, 1987). On the other hand, other studies show that nicotine causes growth retardation and decreases birth weight (Wen et al., 1990; Cliver et al, 1995; Vogt, 2004).

By increasing cigarette smoking in society, especially in young woman, it is necessary to investigate the effects of maternal nicotine exposure during lung development of the offspring. Our previous results indicated that collagen IV expression plays an important role in formation of retina (Nikravesh et al., 2009). Another investigation also revealed that anterior epithelium development and matrix of the lens, especially its marginal zone, are dependent on this molecule (Nikravesh et al., 2009; Jalali et al, 2009). Also, our previous studies (Karimfar et al., 2009; Nikravesh et al., 2009) implicated that the appearance of the collagen type IV during tubule and glomeruli morphogenesis represents that this molecule contribute to nephrogenesis during urinary tract formation (Jalali et al., 2009; Moein et al., 2008). Also, its role in brain choroid plexus (BCP) development indicated that formation of vascular plexus is dependent on collagen type IV, main structural component of the BM (Nikravesh et al., 2009). It seems that factors affecting the collagen regulation during lung development may put the normal health of the respiratory system at risk (West, 2009; Kang et al., 2009; Hinenoya et al., 2008; Lan et al., 2008). Hence, the aim of this study was to investigate the effects of maternal nicotine exposure on lung connective tissue

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development especially collagen type IV, of the mouse offspring.

Materials and Methods

Nicotine administration and tissue preparation

Twenty four female Balbc/c mice were divided randomly into 2 experimental and 2 control groups and appearance of the vaginal plug was designated as day zero of pregnancy. The environmental conditions were 22±1°C and 12 h light-dark cycle with free access to water and food. The experimental group 1 (n=6) injected daily intraperitoneal dose of 3 mg/kg of nicotine from day 5 of gestation to the last day of pregnancy (Hisa et al., 2003). Experimental group (n=6) were received the same amount of the nicotine during gestation and two weeks after birth (lactation). The control groups (n=12) were received nicotine solvent (Normal saline). Finally, the animals were rapidly sacrificed by cervical dislocation and the lung of the mice were removed and fixed for 24 hours at room temperature in formaldehyde 10% and immunohistochemistry study for tracing collagen type IV were carried out.

Immunohistochemistry study

The Avidin-Biotin peroxidase procedure was used for immunohistochemistry study. Sections washed twice for 5 min in 0.05 Tris buffer containing 1.5% NaCl, pH 7.4. For blocking the nonspecific antibody reactions, the sections were preincubated in 0.3% Triton X-100 in TB-NaCl followed by 5% goat serum for 1-2 h. Then they were reacted for 12-24 h at 4°C with primary antibody anti-collagen IV monoclonal antibody (Sigma-Aldrich, USA), diluted 1:50 in TB-NaCl with 0.3% Triton and 2% serum. Tissues were washed with TB-NaCl for three times, each time for 10 min, and incubated for 2 h in biotinylated goat anti-rabbit IgG (1:400 in TB-NaCl). After three further rinses, for 1 h each time, endogenous peroxidase activity was blocked by their incubation in 0.03% H_2O_2 in methanol for 30 min. Tissues were incubated for 2 h in 1:100 avidin-biotinylated horseradish peroxidase complex. Then they were washed three times, each time for 30 min in TB-NaCl, and finally reacted with 0.03% solution of 3, 3-diaminobenzidine tetrahydrochloride for 10-15 min. Tissues containing 0.03% H_2O_2 were washed and lightly counterstained with hematoxylin. Subsequently, they were washed and mounted in PBS glycerol. Collagen reaction in BM of alveolus and lung parenchyma was graded by a sampling computerized method.

Results

Tracing of collagen in different parts of the lung indicated a weak reaction in the alveolar basement membrane in our experimental groups. However this reaction was not significant while compared to the control groups (figures 1a, 1b). These reactions increased to dark brown in the alveolar basement membrane in the experimental groups (table 1) and although collagen synthesis of the BM did not show significant change in experimental groups, the collagen reaction increased remarkably in comparison to the control groups (figure 1c, 1d). Collagen appeared as light brown color in the extracellular matrix in the control groups (figure 2a, 2b). The intensity of reaction in extracellular matrix of lung parenchyma in the experimental groups increased significantly compared to the control groups (figure 2c, 2d). Besides, remarkable signs of picnotic nucleuses and cell death in lung parenchyma in experimental group 2 were observed, but these changes were not noticeable in the experimental group 1 and that of control.

Discussion

Previous studies have shown that ECM and BM play important roles in lung developmental process. Basement membrane is a specialized region of the extracellular matrix consisting of multiple matrix molecules and plays a major regulatory role in developmental phenomena of proliferation, morphogenesis and migration. Among the components of the BM, collagen type IV is the most important parts of this region. Results of this study indicated that collagen increased significantly in the basement membrane (BM) of the lung alveolar in experimental groups as well as lung parenchyma.

These data indicate that although collagen synthesis of the BM did not show significant change in the experimental groups, the reaction remarkably increased. Besides, collagen fibers in the experimental group 2 significantly increased when compared to the experimental group 1 and even some signs of necrosis and cell death in lung parenchyma of experimental group 2 was detectable So, it seems that the lungs of these newborns, exposed to nicotine via the placenta and mother's milk, are more susceptible to damages such as abnormal collagen synthesis and cell necrosis. Our previous studies showed that collagen type IV is a major protein in many developmental processes. The results of this study showed that maternal nicotine exposure leads to collagen changes and basement membrane in the lungs of

their offspring. In mice pups, exposed to nicotine during pregnancy, as well as lactation the collagen fibers showed an increase in basement membrane of respiratory tract and extra cellular matrix.

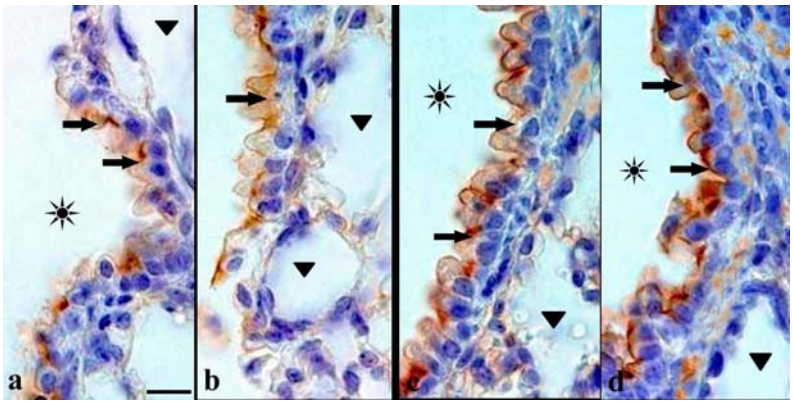

Figure 1. Sections through the respiratory bronchioles of the 14-day old mice that incubated with antibody against collagen type IV in control group 1 (a), control group 2 (b), experimental group 1 (c), and experimental group 2 (d). The respiratory tract lined with columnar epithelium and arrows indicate basement membrane. In these sections terminal bronchiole (asterisks) and lung alveolar (arrowheads) are visible (scale bar=100 µm, Hematoxylin counterstained).

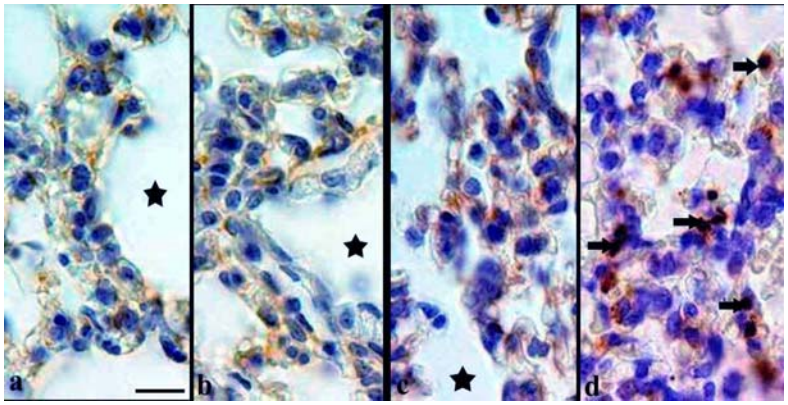

Figure 2. Sections through the lung parenchyma of the 14-day rats. These sections were prepared from control group 1 (a), control group 2 (b), experimental group 1 (c), and experimental group 2 (d) which are incubated with antibody against collagen type IV. The epithelial cells with irregular arrangement and ECM are visible from light to dark brown (arrows). In these sections the terminal bronchiole (asterisks) is visible. Besides to the alveolar cells, cell necroses are obvious (arrows) in the experimental group 2. The micrographs include a scale bar (=100 µm) with Haematoxylin counterstaining.

Table 1. Comparison between lung parenchyma parameters in the experimental and control groups

	Control group 1	**Experimental group 1**	**Control group 2**	**Experimental group 2**
Collagen reaction	**(++)**	**(++++)**	**(++)**	**(++++)**

The intensity of the reaction is assigned by + with the following grades: negative (-), weak (+), moderate (++), strong (+++) and highly strong (++++).

In the exposed animals complications such as cell necrosis in lung parenchyma were also obvious. A cause of this may be the higher level of lipids and acidic property in mother's milk than its serum. As nicotine level in animals, exposed to nicotine via mother's milk, was already shown to be two or three times higher than that of plasma (Gert, 1988).

Studies show a suppression of glycolysis could occurre in lung of animals that are exposed to nicotine. Because type I pneumocytes are dependent on glycolysis and type-II pneumocytes proliferate into type-I ones (Maritz, 1995; Martiz, 1985). It is possible that any change in this phenomenon may lead to a disturbance in programed cell death (Johannes et al., 1998). Although we should not ignore the effect of nicotine on glycolysis, probably nicotine can also induce lipid peroxidation that decreases the antioxidant capacity of the lung. Oxidant/antioxidant imbalance could, in turn, change the genetic program of the genes involved in glycolysis or synthesis of proteins such as collagen type IV.

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Results. This section should describe concisely the rationale of the investigation and its outcomes. Data should not be repeated in both a Table and a Figure. Tables and Figures should be selected to illustrate specific points. Do not tabulate or illustrate points that can be adequately and concisely described in the text.

Discussion. This should not simply recapitulate the Results. It should relate results to previous work and interpret them. Combined Results and Discussion sections are encouraged when appropriate.

Acknowledgments. This optional part should include a statement thanking those who assisted substantially with work relevant to the study. Grant support should be included in this section.

References. References should be numbered and written in alphabetical order. Only published, "in press" papers, and books may be cited in the reference list (see the examples below). References to work "in press" must be accompanied by a copy of acceptance letter from the journal. References should not be given to personal communications, unpublished data, manuscripts in preparation, letters, company publications, patents pending, and URLs for websites. Abstracts of papers presented at meetings are not permissible. These references should appear as parenthetical expressions in the text, e.g. (unpublished data). Few example of referencing patterns are given as follows:

Bongso A., Lee E. H. and Brenner S. (2005) Stem cells from bench to bed side. World Scientific Publishing Co. Singapore, 38-55 pp.

Haddad F., Gholami V. and Pirayesh Shirazi Nejad M. (2009) Ozone inhalation can induce chromosomal abnormalities in bone marrow cells of Wistar rats. Ferdowsi University International Journal of Biological Sciences 1: 41-46.

Tables and Figures. Tables and Figures should be numbered (1, 2, 3, etc.) as they appear in the text. Figures should preferably be the size intended for publication. Tables and Figures should be carefully marked. Legends should be typed single-spaced separately from the figures. Photographs must be originals of high quality. Photocopies are not acceptable. Those wishing to submit colour photographs should contact the Editor regarding charges.

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