Non-enzymatic glycation in diabetic complications

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Diabetes mellitus (DM) is associated with a greatly increased risk of secondary complications, such as cutaneous manifestations, myocardial infarction, nephropathy, retinopathy, and polyneuropathy. The non-enzymatic glycation of proteins followed by the formation of advanced glycation end products (AGEs) plays an important role in the pathology of diabetic complications.[1] Occasionally endocrinological disorders, including DM, manifest themselves by their associated or induced cutaneous abnormalities. A little information is available on what common pathophysiologic theme is responsible for the skin manifestation in diabetes.[2] Therefore, this study was carried out to investigate the extent of *in vivo* glycation of plasma proteins in type 2 diabetic patients with or without cutaneous manifestations. This study also demonstrated the outcome of treatments provided to the type 2 diabetic patients with skin manifestations.

The study was carried out at the District Headquarter Hospital, Faisalabad, Pakistan, and Department of Chemistry and Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad, Pakistan, between November 2005 and March 2006. The Advanced Studies and Research Board (ASRB) of the University granted ethical approval for this study. Seventy-six patients of type 2 DM, and 40 healthy volunteers were recruited for the study after written informed consent. The patients under study were divided into 3 groups; group A consisted of 46 type 2 diabetic patients with skin manifestations, group B consisted of 30 type 2 diabetic patients without skin manifestations, and 40 healthy volunteers formed group C. The research period was divided into 2 phases: phase 1 (pre-treatment phase) and phase 2 (post-treatment phase) had 4 months interval. Different approaches adopted independently by physicians, patients, and caregivers to control type 2 diabetes and its complications were assumed as treatments. A certified dermatologist, nephrologists and retinal specialists carried out assessment of skin diseases, nephropathy, and retinopathy. The post-prandial blood sugar, total plasma proteins, and non-enzymatic plasma protein glycation levels were measured by laboratory kit (enzymatic method) and colorimetric techniques (Hitachi model U-2001 UV/VIS spectrophotometer, Hitachi, Japan). The data were analyzed using the statistical package for social sciences for Windows (version 12.0, 2003, ®SPSS Inc.) with significance level set at $p \leq 0.01$.

The first group of 46 type 2 diabetics (22 women, 24 men) had a mean age of 59 ± 8.83 years (46.60 ± 5.38 years age at onset of diabetes). Among these, 23.9% had retinal complications, while 26.1% presented with kidney problem related to diabetes. Ichthyosiform (shins) (23.7%), scleroderma such as changes of the hands (sclerosis) (17%), diabetic foot ulcers (9%), dermatomycosis (6.6%) and tinea pedis (5.3%) were detected among these patients. The B group comprising 30 type 2 diabetic patients (12 women, 18 men) had a mean age of 54.4 ± 8.0 years (47.67 ± 5.43 years age at onset of diabetes). Retinal (20%) and kidney complications (16.6%) of diabetes were prevalent in those patients. In this study, 56% patients of group A had knowledge of diabetes as compared to 44% of group B. On the knowledge of related complications, equal prevalence (35%) was found for both A and B groups, whereas only 3% had knowledge on management. Statistical difference between the groups when analyzed for age at onset of diabetes was found non-significant (p=0.416).

The mean post-prandial blood sugar of group A and B was initially 13.56 ± 3.16 mmol/L and 11.31 ± 2.39 mmol/L, and after 4 months the levels were 12.28 ± 3.02 mmol/L and 10.76 ± 2.03 mmol/L. Both the groups showed significant improvement (group A: p=0.000; group B: p=0.024) in post-prandial glucose levels however fall of post-prandial glucose at the end of the study was higher in group A, (-1.28 ± 0.14 mmol/L) than in group B (-0.55 ± 0.36 mmol/L). A significant reduction in total plasma proteins (p=0.000) was observed for group A that managed to reduce the mean levels from 14.53 ± 4.37 g/dl to 13.76 ± 4.20 g/dl. Group B showed increasing trend in total plasma proteins as mean value 12.08 ± 3.39 g/dl increased to 12.13 ± 2.97 g/dl. Final assessment after 16 weeks showed a significant difference (p=0.000) in mean levels of non-enzymatic plasma protein glycation (mole of glucose/mole of protein) for group A, from 1.66 ± 0.56 to 1.59 ± 0.52, as compared to slight increase in mean levels of non-enzymatic plasma protein glycation (mole of glucose/mole of protein) for group B, from 1.25 ± 0.56 to 1.29 ± 0.56 (normal range: post-prandial blood sugar = 140 mg/dl (7.77 mmol/L), total plasma protein = 6 g/dl, and non-enzymatic plasma proteins glycation = 0.4 mole glucose/mole protein). In the pre-treatment phase, group A showed a higher mean post-prandial

sugar, total plasma proteins, and non-enzymatic plasma protein glycation than that of group B. Group A also showed statistically significant reduction in all these parameters at the end of study.

The central theme of our study was to determine the non-enzymatic plasma protein glycation in type 2 diabetic patients with and without skin diseases. We also assessed the effect of short-term treatment of type 2 diabetes provided at a public health care center. This study provides an insight into the complex relation between diabetes and skin in Pakistani society, undergoing rapid transition in life style due to industrialization. The thiobarbituric acid method, used for the estimation of non-enzymatic glycation has never been used in clinical trials in Pakistan. It is quite economical to replace costly chromatographical techniques for use at public health care centers. Seventy-six type 2 diabetic patients in our study showed elevated blood sugar and non-enzymatic plasma protein glycation than the control group. These case subjects suffered from renal, retinal, and cutaneous complications. Several articles highlight the role of hyperglycemia and non-enzymatic glycation of proteins in various complications of diabetes.[3,4] Clearly defined in the present study were the effects of diet, exercise, diabetes and/or complications awareness, and physicians treatment criteria that reduced the glycation significantly in group A. This is encouraging since it suggests that beneficial effects may be achieved by therapeutic intervention. Similar to our study, different novel strategies to reduce glycation end-products were reviewed by Soro-Paavonen and Forbes.[1] Group B did not show significant reduction in non-enzymatic glycation, as self-efficacy, and self-management behavior with a high prevalence of limited literacy prevailed in these diabetic patients who were not suffering from any skin disease. This group may need more intensive, culturally appropriate, health education and support. Baseline disease related knowledge in patients with type 2 diabetes, its risk factors, symptoms, related complications, and suitable diet is required to develop risk factor modification interventions, to reduce the impact of long-term complications.[5]

Our study concludes that non-enzymatic glycation of plasma proteins, not only leads to diabetic complications in type 2 diabetic patients, but it is also a measure of (short-term) hyperglycemia, and skin seems strongly associated with the diabetic patient's hyperglycemia. These new observations may broaden the use of skin as an indicator of diabetes and its complications. Further research is necessary to understand how to better facilitate effective and efficient appliance of these intervening assessments in delay or prevention of type 2 diabetes, and policy efforts should be focused on expanding the reach of self-management interventions to the public.

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Possible predisposing factors for thrombotic cerebrovascular accidents in Sudanese patients

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Stroke is a condition that involves loss of brain functions caused by loss of blood supply to part of the brain. It is the third most common cause of death in the developed countries.[1] The etiology of stroke is complex, in up to 40% of affected young adults no underlying cause can be identified.[2] The incidence of cerebral ischemia (CI) in men and women is 6.6-11.3 per 100,000 individuals.[3] In contrast to older patients, hereditary hypercoagulable disorders might play an important causative role in younger patients with CI of undetermined etiology. Well-known, however very rare defects in stroke patients are protein S (PS), protein C (PC), and antithrombin III (AT-III) deficiencies.[4] Among the Caucasian population, factor V Leiden 506 Q is the most common genetic defect causing thrombosis that is currently known.[5] The objective of this study was

to determine the possible predisposing factors for the development of thrombotic cerebrovascular accidents (CVAs) in Sudanese patients.

From 2002-2004, a case-control study involving 100 patients with thrombotic CVAs, and 300 gender and age-matched healthy controls was conducted in the Neurology Department, El-Shaab Teaching Hospital (ETH), Khartoum, and the Department of Clinical Pathology and Immunology, Institute of Endemic Diseases, University of Khartoum, Sudan. The study was approved by the ethics committee. Written informed consent was obtained; patients were selected and admitted to the neurology ward of ETH when they met specific selection criteria: both genders, with confirmed thrombotic CVAs by CT scan of the brain. Patients with known classical predisposing factors such as diabetes mellitus (DM), hypertension, malaria, sickle-cell disease, and so forth, were excluded. Controls were sequentially selected if they do not have obvious medical conditions that could lead to CVAs. Venous blood samples were collected from all patients and controls in ethylenediaminetetraacetic acid, and tri-sodium citrate vacutainer for blood counts (BC), serological, and coagulation assays at the time of the thrombotic event. The AT-III and PC activities were assessed by chromogenic assays, and the PS activities by clotting assay (Diagnostica Stago, Asnieres, France). Screening coagulation assays were performed: prothrombin time (PT), prothrombin-clotting time (PCT), activated partial thromboplastin time (APTT) (Diagnostica Stago, Asnieres, and France). A solid-phase immuno-assay technique was adopted to quantify anti-cardiolipin. The anti-cardiolipin antibodies (ACLA) (immunoglobulin G, immunoglobulin M, and immunoglobulin A) levels <12.5 MPL cut-off point were considered negative for diagnosis of CVAs. The LA was evaluated using a coagulation mixing experiment. Screening enzyme-linked immunosorbent assay test was adopted to quantify the anti-phospholipid antibodies (APLA) using platelets concentrate products.

Means were compared using Epi info program version 2002. The mean standard deviation age of the patients and controls were 32.0±2.0, and 33.0±3.0 years (p=0.91). Females were more affected than males, with an M:F ratio of 1:3. By direct questioning, 60% of women with CVAs in our study were on oral combined contraceptive pills. There were no significant differences in the means of hematological parameters (hemoglobin [p=0.062], packed cell volume [p=0.069], red blood cells [p=0.34], white blood cells [p=0.23], platelets [p=0.066], and erythrocyte sedimentation rate [p=0.11]) of patients compared to controls (p>0.05) (**Table 1**). There were no significant differences in the means of PT (p=0.063), TCT (p=0.063), and APTT (p=0.084) of patients compared to healthy controls (p>0.05). The LA was found negative. The mean levels of APLA (p=0.6) and ACLA (p=0.056) were below cuff-off point of 0.16 for patients (p>0.05), and 9.4 for controls (p>0.05). The PC mean level in patients was at the lower normal range [NR]: 70-130%, with a mean 60±4% (NR: 55-64%; p=0.04). While the controls' mean level was found to be significantly higher than that of the patients' (mean 122.5±3%, NR: 120-130%; (p=0.04). The PS mean level of the patients was at the lower NR (65-140%) with a mean 61±4%, NR: 55-65%. While the controls mean level was found to be significantly higher than that of the patients' (mean 125±10%, NR: 115-135%; (p=0.03). The AT-III mean level in patients was at the lower NR (80-120%) with a mean 55±2%, NR: 53-57%. While the controls mean level was found to be significantly higher than that of the patients' (mean, range 90-110%; p=0.02).

Stroke in Sudanese patients is an important cause of disease and disability; however, no systematic study has been conducted to elucidate possible causes. Generally, CVA in young individuals is rare, however possibly alarming. Classical risk factors for development of CVAs are more prevalent among older subjects. In this study, the hematological profiles for patients were within normal range. With normal ranges of APTT and thrombin clotting time, LA and dysfibrinogenemias can be excluded. Natural anticoagulants (PC, PS, and AT-III) were at the lower range of normal in all patients (p=0.04), and were significantly lower from those of the age (p=0.04), and gender matched controls (p=0.02). In addition, most of the female patients were on oral contraceptive pills at the time of the stroke. Female patients had even lower AT-III levels compared

Table 1 - Hematological profiles of the study groups.

Variable	Age (years)	Hb (g/dl)	PCV (%)	RBCs $X10^{12}$/L	TWBCs $X10^{9}$/L	Plts $X10^{3}$/L	ESR mm/h
Patients (n=100)	32 ± 2	11.1 ± 0.3	26 ± 2	4.1 ± 0.2	5.3 ± 0.1	170 ± 20	7 ± 2
Controls (n=300)	33 ± 3	14.1 ± 0.2	46± 5	5.2 ± 0.3	6.3 ± 0.2	385 ± 69	8 ± 3

Hb - hemoglobin, PCV - packed cell volume, RBC - red blood cells, TWBC - total white blood cells, Plts - platelets, ESR - erythrocyte sedimentation rate, all values expressed are not significant.

to female controls. In women taking contraceptive pills, the coagulation derangement caused by the pills probably increased the thrombotic potential of the low normal levels of the natural anti-coagulant proteins. In conclusion, relative PC, PS, and AT-III deficiencies could be the possible causes of the increased incidence of CVAs in Sudan. Additional external factors like contraceptive pills or dehydration that accompanies some diseases could be implicated. Further studies are needed to verify our suggested explanation.

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Autologous serum is more effective than fetal bovine serum on proliferation of bone marrow derived human mesenchymal stem cells

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Human mesenchymal stem cells (hMSCs) are non-hematopoietic pluripotent stem cells which can differentiate into cells, tissues, or both, originating from all 3 germ layers, primarily the bone, muscle, cartilage, tendon, fatty, neural, and bone marrow stromal cells. Despite the fact that bone marrow represents the main available source of hMSCs, they can also be isolated from many other tissues. The hMSCs have the potential of clinical use in many fields, primarily hematopoietic stem cell transplantations, tissue engineering, and gene treatments progressively increases the interest in these cells. Mesenchymal stem cells (MSCs) are used for promoting engraftment, and to prevent and treat the graft versus host disease in allogeneic stem cell transplantations.[1] Furthermore, clinical practices with hMSC therapies have been initiated in certain neurological diseases such as amyotrophic lateral sclerosis. Clinical trials have achieved a modest degree of improvement in cardiac function with autologous bone marrow derived stem cell therapy. Fetal bovine serum is added to the culture media for the production, proliferation, and differentiation of the MSC. In clinical practice, the use of fetal bovine serum carries a potential risk for viral infection, prion transmission, and variant Creutzfeldt-Jakob disease. The other limitations regarding the use of fetal bovine serum in clinical practice include the immune reactions occurring due to contamination with bovine proteins. As a result of these immune reactions, development of antibodies to bovine proteins occurs, and particularly with repeated applications, the engraftment of hMSCs fails or a subsequent rejection may develop even if the engraftment is achieved. Addition of autologous serum to culture medium instead of fetal bovine serum may be an alternative approach to overcome these problems. The aim of this study is to compare the effects of the autologous serum and the fetal bovine serum on the proliferation of hMSCs. The demonstration that autologous serum can provide a hMSC proliferation as effective as that obtained with fetal bovine serum would motivate comprehensive studies on the subject and enable safer applications in clinical practice.

The study was carried out at the Faculty of Medicine, Karadeniz Technical University Hospital in Trabzon, Turkey, between January 2004 and June 2005. Heparinized bone marrow aspirate was obtained from 3 healthy volunteer donor candidates of allogeneic bone marrow transplantation during the routine bone marrow examination performed after receiving consent from the donors and the local ethical committee. The donors mononuclear cells were isolated by ficoll density gradient method. The collected cells were at least 2 times centrifuged with serum free culture medium (Roswell Park Memorial Institute [RPMI]-1640) of 5-fold volume. After these procedures, the prepared mononuclear cell suspension rich in hMSC of bone marrow origin was prepared for the assay. For autologous serum, 100-cc of peripheral venous blood was obtained from the donors from whom bone marrow was taken,

sera were separated after centrifugation and frozen at -20°C in 2-cc plastic tubes. When required, the frozen autologous serum was thawed in an incubator at 37°C before use. Each phase was performed in triplicate with bone marrow aspirates obtained from the 3 different individuals in accordance with the following assay design. Primary culture procedures were initiated by cultivating the isolated mononuclear cells of bone marrow origin into 2 separate 25-cm^2 plastic flasks so that there were 1 x 10^7 cells in each flask. As culture medium, RPMI-1640 + 10% autologous serum and RPMI-1640 + 10% fetal bovine serum were added to the first and second flasks, with the intention to compare the effects of the autologous serum and fetal bovine serum on the proliferation of hMSCs. The nourishment of the cells that adhered to the flask as from the third day of the culture was continued on a twice-weekly basis. After the cells covered the whole base of the culture dish, the primary culture was completed. The immunophenotypical analyses of the cells obtained by trypsinization were performed and the procedures of the first passage culture were initiated. After the primary culture procedures, the first passage culture procedures were initiated following the disassociation of the cells from the plastic flasks by trypsinization. For the first passage culture, 1 x 10^6 hMSCs were placed in the same 2 x 25-cm^2 plastic flasks to be nourished with autologous serum and fetal bovine serum, and similarly, they were left for proliferation until they covered the base of the flask with nourishment continued on a twice-weekly basis. After the cells covered the whole flask base, the first passage was terminated.

The study was performed as a result of a 3-stage process. At the first stage, the mean rates of the cells covering the flask base at the end of the primary culture and first passage culture were determined by 3 independent persons with an inverted microscope (as %) in the cell groups nourished with autologous serum and fetal bovine serum and the values compared. At the second stage, the antigenic variation occurring in the MSC within the culture process was investigated in an in vitro setting and the effects of the autologous serum and fetal bovine serum on this variation were compared. At third stage, methyl tetrazolium test (MTT) was performed to compare the effects of the autologous serum and fetal bovine serum on hMSC proliferation. For this purpose, the MSCs obtained by trypsinization after first passage culture were cultivated in the 96-well plastic plates in a such a way that there were 1 x 10^4 cells in each well. Two 10-well groups were formed and one group was incubated with RPMI + 10% fetal bovine serum and the other with RPMI + 10% autologous serum for a period of 10 days. After the incubation, the cell viability was analyzed by MTT test and the data were compared. Mann Whitney-U test was used for intergroup comparisons and $p<0.05$ value was considered statistically significant.

On the 10th day of primary culture, the cells incubated with medium containing autologous serum were observed to cover the whole flask base whereas the cells incubated with fetal bovine serum were detected to proliferate to an extent to cover 50% of the flask base. The results obtained as a result of the first passage culture procedures carried on for 10 days were similar to those of the primary culture. These results show that autologous serum provides a more effective proliferation compared to fetal bovine serum for production of hMSCs from mononuclear cells of bone marrow origin. The MTT test was performed to compare the effects of the autologous serum and fetal bovine serum on the proliferation of the hMSCs. The optical density of the cells was 257±29.7 in the autologous serum group, and 140±19.7 in the

Table 1 - Immunophenotype of human mesenchymal stem cells (hMSCs) after primary culture and first passage culture.

Surface antigen	After primary culture (n=6)		After first passage culture (n=6)	
	AS group	FBS group	AS group	FBS group
CD45	11.9 ± 4.2	19.2 ± 5.2	1.7 ± 1.6*	9.1 ± 3.5
HLA-DR	28.5 ± 13.2	47.3 ± 14	1.7 ± 2.2*	10.9 ± 7.6
CD34	11.7 ± 6.5	15.2 ± 3.4	5.2 ± 3.8	9.2 ± 6.8
CD49	17.8 ± 3.6	17.7 ± 4.5	15.5 ± 11.4	19.4 ± 1.9
CD14	14.1 ± 1.8	18.6 ± 5.6	1.4 ± 0.8*	5.2 ± 3.6
CD166	81.7 ± 9.6	82.2 ± 1.2	98.0 ± 1.3	90.9 ± 5.4
CD105	88.5 ± 9.8	84.6 ± 6.4	95.6 ± 4.0	89.4 ± 4.5

AS - autologous serum, FBS - fetal bovine serum, CD45, CD14, and HLA DR expression are higher with fetal bovine serum added to the medium groups than with autologous serum added to the medium groups at the end of the first passage culture, values are expressed in percentage ± standard deviation, *$p<0.05$ AS versus FBS groups.

fetal bovine serum group. The intergroup difference was considered statistically significant (p=0.001). Similar to the morphological evaluation, the results obtained from the MTT test indicated that autologous serum provided a more effective hMSC proliferation compared to fetal bovine serum. As a noticeable result with respect to immunophenotypical characterization, the groups with fetal bovine serum added to the medium exhibited higher rates of CD45, CD14, and HLA DR expression both at the end of primary culture and first passage, compared to those with autologous serum added to the medium. However, the differences between the 2 groups were detected to reach a level of statistical significance only at the end of the first passage culture (**Table 1**). In addition, the cells incubated with fetal bovine serum revealed a less positive expression of hMSCs markers such as CD105, CD166, and CD29. However the difference between the groups was not statistically significant.

Autologous serum may be an alternative to fetal bovine serum due to the limitations resulting from the use of fetal bovine serum in the stages of hMSC proliferation and differentiation in clinical practice. In this study, the effects of the autologous serum and fetal bovine serum on the hMSC proliferation were investigated. Based on the data obtained, the proliferation of the hMSCs was detected to be statistically significantly faster in the autologous serum group. The fact that autologous serum provides a faster mesenchymal stem cell proliferation compared to fetal bovine serum may be due to the higher transforming growth factor-β, platelet derived growth factor, basic fibroblast growth factor, and endothelial growth factor content of the human serum. In the literature, there are few studies comparing the effects of the autologous serum and the fetal bovine serum on the MSC proliferation. Stute et al[2] reported that autologous serum and fetal bovine serum had similar effects on hMSC proliferation. The results obtained from the studies comparing the effects of autologous serum and fetal bovine serum on the cell proliferation in different human cell series are controversial. While Shigeno and Ashton[3] suggested that autologous serum was superior to fetal bovine serum for human bone cell proliferation, Anselme et al[4] and Yamamoto et al[5] demonstrated that autologous serum and fetal bovine serum had similar effects on the proliferation of the bone marrow cells. There is plenty of data concerning the immunophenotypical characteristics of bone marrow originated hMSCs, which are grown in culture media. Cultured MSCs cells do not express hematopoietic markers such as CD34, CD14, and CD45. Markers accepted to be typical for MSCs are SH2 (CD105), SH3 (CD73), and SH4 (CD73). When we compared the antigenic characteristics of MSCs grown in autologous and fetal bovine serum groups, we saw that CD105 expression, which appears to be one of the most typical markers for MSCs, was expressed more in the autologous serum group, both in primary culture and at the end of the first passage. The difference in the 2 groups was not statistically significant, probably due to the small number of examinations (3 trials). In the fetal bovine serum group, it was detected that markers of hematopoietic origin, such as CD34, CD14, CD45 and HLA-DR were expressed more both in primary culture and at the end of the first passage, compared to the autologous serum group. The CD14, CD45, and HLA-DR expressions were statistically significantly higher in cells fed with fetal bovine serum, particularly in the cells obtained from the first passage. The higher expressions of CD34, CD14, CD45, and HLA-DR in fetal bovine serum group suggested that the homogeneity of MSCs developed by fetal bovine serum was lower compared to the autologous serum group, and that the hematopoietic cells survived in higher proportions. This result may be due to the different growth factor and cytokine content of fetal bovine serum, while on the other hand it may be related to causing more hematopoietic cell proliferation, so that it contains foreign antigenic proteins. Another assumption is the possibility that the foreign antigenic stimuli in the fetal bovine serum may induce hematopoietic cell differentiation from MSCs. The higher expression of CD105 and lower expressions of CD34, CD14, CD45, and HLA-DR in the autologous serum group suggests that autologous serum is more convenient for hMSC proliferation, compared to the fetal bovine serum.

In conclusion, the use of autologous serum instead of fetal bovine serum may provide a faster proliferation of MSCs of bone marrow origin. Results demonstrate that in clinical MSC practices, autologous serum may be used instead of fetal bovine serum for MSC proliferation.

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Effects of *crocus sativus* on the fetal development of NMRI mice

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Crocus sativus (CS), commonly known as saffron, is a native Iranian herb, which is commonly used in Folk medicine, in Iran, India, and the Middle East for flatulent colic, to increase appetite, to relieve abdominal pain, to promote menstrual bleeding, antisecretory, antiulcer activities, and as an aphrodisiac,[1] in addition, it is necessary to mention that this herb is used vastly in cooking by Iranian people as an additive in foods. *Crocus sativus* is known to contain various chemical constituents including crocin -1, Picrocrocin, staytry, vitamins B1, B2, fixed oils, carotenoids, colchicine, Quercitin, proteins, wax, and mucilage. It was reported that the extract of CS has an antitumor, anticarcinogenic effect, inhibits colony formation, and nucleic acid synthesis by malignant human cells.[2] There is an imperative for researchers to determine whether herbal compounds, particular those used for cooking, have any adverse affects on fetal development. This paper presents the results of a study designed to address whether there is a risk of adverse fetal outcome with the use of CS during pregnancy.

This study was carried out in Gorgan University of Medical Sciences in Northern Iran during 2006. *Crocus Sativus* was purchased from a famous cultivated commercial brand (Novin zaferan Company) in Mashad, Iran. A voucher specimen (159-0319-02) was designated for the CS in this investigation by the Mashad University of Medical Sciences in Khorasan province in North-East Iran. The stigmas were collected in autumn and air dried at 40°C. The saffron powder was dissolved in 60 cc water. The solution was kept in the dark for 4 days. The prepared solution was filtered and was concentrated at 30-40°C temperature using a rotator. The extraction rate was 5% and the final concentration of aqueous extract was 28%. The high performance liquid chromatography analysis of CS extract using ultra violet detector with wavelength of 308 nm showed the presence of Safranol with a concentration of 5.1%. The pure Safranol (Fulka Chemical Corporation, U.S.A) was used as a standard to determine the Safranol concentration in CS (Saffron) extract. Fresh saffron was provided each day. The animals used in this study were experimentally, 28-30 gram weighted and 7-8 week old virgin female and mature male inbred mouse strain (NMR-I). The males were part of the animal house breeding stock with confirmed mating experience. Dry food pellets and water were provided ad libitum with animal house conditions maintained at 20.1-21.2°C, 65.5-65.8% relative humidity, and a 12 h: 12 h photoperiod (lights on 0700-1900h). Approval for this study was gained from the Gorgan University of Medical Sciences Animal Care and Ethics Committee. Two females were caged with a male of the same strain overnight. The presence of vaginal plug the next (following) morning confirmed that mating had taken place and was designated as day zero of pregnancy (Gestation Day 0: GD 0). Females that did not mate within 2 estrus cycles were excluded from the study. Also, maternal weight was measured throughout the experiment. Pregnant mice were randomly divided into experimental and control groups. Ten mice in each group orally received 100 mg/kg/day CS extract and normal saline, from GD 6 to GD 15, by oral intubation. On GD 18, the pregnant mice were sacrificed under ether anesthesia and the uterus was opened and the umbilical cord cut close to the fetus; each fetus and placenta was then weighed. Each fetus was assigned a number according to its position in the uterine horn, starting with number one at the ovarian end of the left uterine horn. Fetuses were assessed as either alive or dead and any resorptions noted; live fetuses were then euthanased by hypothermia. All live fetuses were measured biparietal diameter (BPD) crown-rump length (CRL), and examined externally for malformations or deviations from normal growth as described. Also, each of the fetuses was weighed by sensitive electronic measurement serrations (GT 210 Ohaus Co, USA) and observed by stereo research microscope, Olympus SZX Japan. Fetuses were eviscerated and the skin removed to facilitate stain penetration. Skeletal staining of fetuses was performed by the Alcian Blue-Alizarin Red S method. Descriptive statistics were calculated for water consumption. Differences in body weight, BPD and CRL between control and treatment group

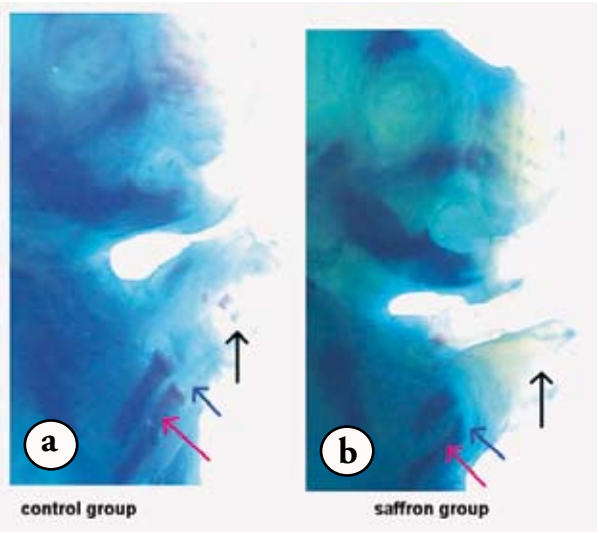

Figure 1- Gross appearance of skeletal staining of fetus in **a**) control and **b**) saffron group, Black arrow: comparing delayed ossification in saffron groups. Red arrow: ossification area, Blue arrow: cartilage area.

were analyzed using a one-way ANOVA with Tukey post-test. Correlations between fetal weight, placental weight, and crown-rump length were carried out using the Spearman Rank Order Correlation coefficient. Resorbed implantation frequency was tested using an x^2 analysis.

During the whole experiment, no maternal deaths and behavioral changes were recorded in any group. The CS-treated females consumed as much food and water as the controls and gained comparable weight. There were no signs of maternal toxicity due to CS treatment. No signs of toxicity were noted in any of the animals. All pregnant animals appeared healthy at sacrifice. One hundred and twelve fetuses were derived from controls and 97 fetuses derived from treatment groups. The mean of fetuses per one mouse was 11.25 in controls and 9.7 in treatment groups. The weight of fetuses in case (0.9633 ± 0.482) was lower than controls (1.1265 ± 0.113) (p<0.05). Placenta weight in the treatment group significantly decreased (0.22±0.08 versus 0.30±.0.08) (p< 0.05). Also BPD in the treatment group (6.7660 ± 0.53) was significantly higher than the control (6.5023 ± 0.293) groups (p<0.05). Crown Lump length in 2 groups were similar. Implantation and number of fetuses in the left horn (55%) of uterine of the treatment group was higher than the right horn (45%). While in the control group this percent was similar, 48% in the right and 52% in left the horn of uterine. Correlation between placenta weight and fetal weight in the treatment group was significant. On the other hand, lower placenta weight lead to lower weight in the CS group. Also, a positive correlation was observed between increasing of BPD with CRL in the treatment group. Major congenital malformations in fetuses were not found in both treatment and control groups. After Alizarin red S and Alcian blue staining in the treatment group, the extract of CS caused delayed bone ossification in hip, metatarsus, metacarpus, fingers, and sternum in all stained fetuses (**Figure 1**). The reduction of Alizarin staining of the hyoid bone was found in all experimental and controls groups. The missing clavicle bone was observed in one specimen from the experimental group.

The examination of skeletons of crocus-exposed fetuses showed partial ossification of the supraoccipital bones. The other bones of the skull, including basicranial ones, did not show any malformation. Also, partial ossification was seen in the vertebral column, while upper vertebra such as cervical, thoracic, lumbar ossified normally, lower vertebra such as sacrum and coccyx partial ossified. In the limbs, delayed ossification was observed in distal bones such as metatarsal, metacarpal bones, and phalanges. Shoulder girdle bones were observed as normal. Pelvic girdle partial ossification was observed in the ischium bone.

The findings of this study showed that the aqueous CS extract can induce delay in bone ossification (BO) in mice fetus if it is used continuously during the embryonic period. However, CS extract did not cause any major birth defect in fetuses. There are very rare studies on the teratogenic effect of CS. The Martin study[3] on xenopus showed that the high concentration of crocetin, one of the compounds of the CS extract has a teratogenic effect on xenopus fetuses. Martin et al[3] indicated CS saffron has a teratogenic effect, however, in their study they noted that the concentration of crocetin must be higher than 100 micrograms to cause a teratogenic effect.[3] The exact mechanism of developmental toxicity of CS is not clear. One possible mechanism is related to the compound of this plant. Escribano[4] in a study indicated that crocetin of CS has a blocker effect on cell growth, by preventing osteoblast cells proliferation, the plant can cause delay of bone ossification. Escribano indicated that the crocetin of CS has an inhibitory effect on cells growth; it inhibited the synthesis of nucleic acids in cells.[4] Also, it was reported that the extract of *crocus sativus,* which contains carotenoids, had an antitumor effect and inhibited colony formation and nucleic acid synthesis by malignant human cells.[2] In this study, the fetuses of the treatment group had decreasing weight and increasing BPD. It may be related to BO of the skull bone. The decreasing weight can be due to reabsorbing of extra cellular liquid in the fetus.[2] The growth of the fetus during intrauterine life is reflected in the weight at birth. Fetal growth is largely determined by the availability of

nutrients from the mother, as well as placental capacity to supply these nutrients in sufficient quantities to the fetus. Of course there is evidence that both placental volume and the rate of placental growth may influence fetal size. Skeletal development is used as a sensitive marker of alterations in overall fetal development. This study indicated that administration of 100 mg/kg/day of CS extract during the embryonic period can cause delay of BO, however, in this dosage this herb could not cause major birth defects in fetuses. The scale and frequency of bone malformation presented in this study such as poorly ossified vertebras, together with metacarpal and metatarsal delay ossification, are comparable to results observed in fetuses exposed prenatally to caffeine and to paracetamol.[5] However due to the different chemical structure, mechanism of action, and metabolic pathways of caffeine, paracetamol and CS, this kind of analysis is purely theoretical.[5] Delaying of BO due to CS may be due to the blocker effect of this herb on cell growth; it means that this plant by preventing osteoblast cell proliferation can cause delay of BO. Regarding the concentration of CS saffron in this study, and Martin's results on xenopus, the lack of birth defects in this study may be due to the concentration of CS. Therefore, we suggest that pregnant women avoid high consumption of CS (saffron) during the organogenesis period, although further studies to determine the exact mechanisms of developmental toxicity of CS are needed.

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Prevalence of smoking among female medical students in the College of Medicine, Riyadh, Saudi Arabia

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Tobacco epidemic in women is growing in Asia and tobacco companies promote the false perception of cigarettes as both fashionable and a weight control aid.[1] Despite the well known hazards of smoking, it is still increasing.[2] In 1999, 23.2% of Japanese women (20-29 years) admitted to smoking, while that figure was only 10.5% in 1986.[1] Not only young and old age groups are affected but also teenage smoking is of immense concern.[1] Girl's figures exceed that of boys. According to WHO figures, in 1994, 27% of Spanish 15-year-old girls smoked, while only 20% of boys the same age did.[1] In Sweden, 19% of 15-year-old girls smoked whereas only 15% of the boys the same age did.[1] Despite it being illegal for people under age 20 to smoke, 4.3% of teenage girls now smoke in Japan.[1] The situation of male smokers in the Kingdom of Saudi Arabia (KSA) may not differ much from other countries. Smoking prevalence among secondary school boys was found to be 21.8% in one study.[3] A recent study has shown a smoking prevalence of 13% among male medical students in Riyadh.[4] Most if not all of the previous studies in KSA were conducted among males. To obtain an estimate of smoking prevalence in young females, this study was conducted among female medical students in the College of Medicine, King Saud University (KSU), Riyadh, KSA.

A self-administered questionnaire was distributed during October 2006 to all female students in the College of Medicine, KSU, Riyadh (n=447). The majority of questions have yes or no answers in a one-page questionnaire. Three hundred and thirty-seven students responded (75.4%). Smoking behavior was categorized into: current smoker, anyone who smoked some kind of tobacco product everyday; ex-smoker, anyone who quit smoking for one month or more; and never smoke, anyone who never smokes in their life. Data were analyzed using SPSS version 10. Informed verbal consent was obtained from the students. The prevalence of current smoker was 2.4% while, ex-smoker

Table 1 - Prevalence of smoking among female students, College of Medicine, King Saud University, Riyadh, KSA (n=337).

Smoking status	Frequency	(%)
Current smoker	8	(2.4)
Ex-smoker	12	(3.6)
Never smoke	316	(93.8)
Missing (not answered)	1	(0.3)
Total	**337**	**(100)**

represented 3.6% of the participant's (Table 1), most were smoking Shesha (70%). Influence of friends has a strong effect on initiating smoking in 30% of smokers, while another 30% state no specific reason for starting smoking. Only 10% blame their smoking parents as a reason for them to initiate smoking. Three percent of students were passive smokers (n=10). Almost all smokers were motivated to stop smoking.

The present study showed that the prevalence of active smoking among female students in the College of Medicine, KSU was 2.4% which was less than the prevalence of smoking among male medical students (13%) which was carried out in the same medical college in 2005.[4] All previous studies carried out in KSA showed that smoking is a common health problem among male adolescent and students at different age group.[3-5] The prevalence of smoking among female students was not studied widely in KSA, due to the sensitivity of questioning females on smoking, which is prohibited by Islamic law. However, this study showed that smoking is present among female students (smoker 2.4% and ex-smoker 3.6%). As the tobacco industry increasingly targets Asian women with messages equating smoking with equality and personal freedom, we will experience a young women's smoking crisis unless we act now to prevent it.[1] Globally, 4.9 million deaths each year are attributed to tobacco use, and this may increase to 10 million within the next 20-30 years, of these deaths, 70% are likely to occur in developing countries.[6] This study showed that more than 70% of female smoking medical students smoked Shesha. In comparison with the study, which has been carried out among male medical students, it showed that 44.1% of male smoker students smoked Shesha, and 23.7% smoked both cigarettes and Shesha.[4] Hubble-bubble prevalence is increasing, especially among women, but public awareness of its risk factors is virtually nonexistent or still premature. In general, there is misconception that Hubble-bubble smoking is a safe alternative to cigarette smoking. Much work remains to be carried out to adequately define the hazardous effects of Hubble-bubble smoking. Tobacco use is an important public health issue in KSA not only for adults but also for adolescents. Moreover, the prevalence of smoking among adolescents appears to be rising, with more children and adolescents becoming regular users of tobacco each day.[7] Sitting with friends who smoke was the main reason for smoking among female smokers in this study. Another study which has been carried out in the same college among male medical students showed that the common reason for the smoking behavior was the influence of friends.[4] This study emphasized the need for a health education program for smoking cessation in the medical college as data showed that approximately 90% of female medical students in this study did not notice any health education program in their medical college. Another study showed that male medical student's opinion regarding the availability of health education activities in the medical college were as follow: always available (3.4%), not available (54.7%), and sometimes available (29.8%).[4] There is an urgent need from all health teams members to place more emphasis on the promotion of non-smoking campaigns in the community. It should be mentioned that, this study may not reflect the real prevalence of smoking among female medical students in KSA as it was conducted only in one college. Also, the sensitive issue of smoking may prohibit some smokers from acknowledging this habit.

In conclusion, smoking habit is a present health problem among female medical students that needs further exploration and planning for effective health education program in medical college.

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