Effects of vitamin D in lung, stomach, esophagus and testis tissues following administration of urethane in balb/c mice

Fariba Koohdani, MSc, PhD, Jalil Baghdadchi, MSc, PhD, Farhang Sasani, DVM, PhD, Kazem Mohammad, MSc, PhD, Parvin Mehdipour, MSc, PhD.

ABSTRACT

الأهداف: توضيح دور فيتامين D في العملية التبادلية وتحول (exon 1) في(K–ras) في الأنسجة بما فيها الرئة والمعدة والمريء والخصية بواسطة عقار يوريثين.

الطريقة: أجريت دراسة تجريبية على الفئران المستولدة نوع balb/c وتتراوح أعمارها بين 11–9 أسبوع واجري لها التشخيص. أجريت هذه الدراسة في الفترة ما بين 2003م الى 2005م بقسم الجينات الطبية وقسم العلوم الطبية بجامعة طهران. تم تصنيف العينات الى ثلاث مجموعات: تم تصنيف أول مجموعة بواسطة الحقن باليوريثين لثلاث مرات (600 ملجم/كجم/اليوم لمدة 48 ساعة). أعطيت المجموعة الثانية 3.5 ملجم/ملجم/ 1000مللتر) من فيتامين D في ماء الشرب لأربعة أسابيع بعد تناول يوريثين كما في المجموعة الأولى. وكانت المجموعة الثالثة مجموعة التحكم. تم التضحية بجميع الفئران بعد 20 أسبوع بعد ذلك تمت إزالة الأنسجة وإجراء الفحص لمتغيرات الأنسجة المرضية وتحولات المحور العصبي للجين K–ras

النتائج: تمت دراسة كامل عدد الفئران الثلاثين. كان التشكل لورم الرئة قد ازداد بشكل ملحوظ في مجموعة اليوريثين وذلك بالمقارنة مع مجموعة التحكم ($p<0.005$)، ولكن لم يتم إيجاد مثل هذا الفرق في مجموعة (U+D) ومجموعة التحكم. بالإضافة الى أنه لم يكن هنالك فرقا ملحوظاً بين جميع المجموعات في الأنسجة الأخرى التي تم فحصها. لم يكن هنالك تحول في(exon 1) للجين (K–ras) لأورام الرئة والأورام الغدية الحرشفية والتبدل الكامل في المعدة.

خاتمة: أظهرت نتائجنا أثر مضادات الأورام الجينية لفيتامين D3 في أورام الرئة بما في ذلك عقار يوريثين. قد ينقص فيتامين D خطر الأورام الوراثية للحمية التي تحتوى على أطعمة ذات نسبة عالية من الخميرة والمشروبات التي تنتج اليوريثين في عمليتها.

Objectives: To elucidate the role of vitamin D in the histopathological alterations process and K-ras gene mutation (exon 1) of tissues including lung, stomach, esophagus, and testis, by the administration of urethane.

Methods: An experimental study in inbred balb/c mice aged 9-11 weeks was designed. This investigation was performed from 2003 to 2005 in the Department of Medical Genetics, Medical Sciences/University of Tehran, Tehran, Iran. The samples were classified into 3 groups: the urethane group was characterized by intraperitoneal injection of 3 times urethane (600 mg/kg/day at 48 hour intervals). The second group (U+D) was given 3.5 mg/kg (6.3 mg/1000 ml) vitamin D in the drinking water for 4 weeks following the same intake of urethane as the first group, and the third one was the control group. All mice were sacrificed after 20 weeks, tissues were removed and examined for histopathological changes and mutations in the exon 1 of the K-ras gene.

Results: Thirty mice were studied. The formation of lung tumor was, significantly, increased in the urethane group as compared with the control group ($p<0.005$), however, such a difference was not found in the U+D and control groups. In addition, there was no significant difference between all groups in other examined tissues. There was no mutation in the exon 1 of K-ras gene of the lung adenomas, adenocarcinomas, and stomach metaplasia.

Conclusions: Our results showed the anti-tumorigenic effect of vitamin D3 in lung tumors induced by urethane. Vitamin D may reduce the risks of a tumorigenic diet that includes high fermented foods and beverages that produce urethane in their process.

Saudi Med J 2008; Vol. 29 (3): 340-344

From the Departments of Community Medicine (Koohdani), School of Medicine, Pathology (Sasani), Faculty of Veterinary, Epidemiology and Biostatistics (Mohammad), School of Public Health and Institute of Public Health Research, and Medical Genetics (Mehdipour, Koohdani), School of Medicine, Medical Sciences, University of Tehran, and the Department of Nutrition (Koohdani, Baghdadchi), Faculty of Health and Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran.

Received 14th August 2007. Accepted 9th January 2008.

Address correspondence and reprint request to: Dr. Fariba Koohdani, Department of Community Medicine, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran. Tel. +98 (912) 2182802. Fax. +98 (21) 66404377. E-mail: fkoohdan@tums.ac.ir

Sunshine and vitamin D are protective factors for cancers in many organs.[1] Previous studies have shown that 1,25-Dihydroxyvitamin D3 [1,25-(OH)2D3] and its analogs are able to reduce the invasiveness of metastatic cancer cells in vitro. Several studies have focused on the influence of vitamin D3 and its analogs on the metastasis of lung cancer in a defined animal model.[2,3] In the present investigation, the carcinogen group (U) was injected with urethane. The United States food and drug administration (FDA) nominated urethane for study because of the widespread exposure of humans through the consumption of fermented food and beverages such as wine and bread. It is anticipated to be a carcinogen in humans.[4] On literature review, we were unable to find studies on the anti-tumorigenic effect of vitamin D in tumors induced by urethane. The K-ras mutations are commonly observed in human lung adenocarcinomas, and in mouse lung tumors initiated in fetuses or adults. They are early lesions found in hyperplasias as well as advanced cancers.[5,6] The role of K-ras mutation in lung tumors induced by urethane is controversial and requires further studies.[6,7-10] We aimed to study the interfering effects of oral administration of vitamin D3 preceding with the injection of urethane in lung, stomach, esophagi, and testis tissues of balb/c mice and their influence on the exon 1 of the K-ras gene of proliferative lesions.

Methods. This investigation was performed from 2003-2005 in the Department of Medical Genetics, Medical Sciences/University of Tehran, Tehran, Iran and approved by the ethics committee of the Medical Sciences/University of Tehran and Tabriz University. Thirty inbred balb/c mice (15 male and 15 female), with age range of 9-11 weeks old, and weights of 12-15 gm (female) and 17-20 gm (male) at the commencement, were housed in plastic cages with stainless steel wire lids (5 mice per cage). The animals were classified into 3 groups, 10 mice per group (5 mice per gender) and they were matched for age and weight within each group. The animals received standard food pellets and drinking water ad-libitum. The consumption of drinking water was measured in each group. After 2-weeks acclimatization, mice of 9-11 weeks age, as the normal control group (C) received only drinking water every day during the treatment period, while the carcinogen group (U) was injected intra peritoneally (IP) with 600 mg/kg of urethane (C3H7NO2, MW: 89, LOT: 125 H03/8 from Sigma company, Saint Louis, MO, USA) in 0.9% sodium chloride (NaCl) 3 times at 2-day intervals. The treated group (U+D) was administered 3.5 mg/kg vitamin D3 (C27H44D, MW: 384.65, Code No: 500936 from Merck company, Darmstadt, Germany) orally by drinking water for 4 weeks starting simultaneously with injection of urethane. Vitamin D intake measured per animal according to the average animals weight and water consumed per each of the cages. During the intervention 4 mice died, all the surviving mice were sacrificed 20 weeks following IP injection of urethane 1% (0.5-1.2 ml). Their lung, stomach, esophagi, and testis were removed and a portion of them was fixed in 10% neutral buffered formalin for histopathological diagnosis and the remaining was stored at -70°c for detection of K-ras mutation. Then the fixed tissues were embedded in paraffin. The paraffin blocks were sectioned at approximately equal thickness (5µ) by LEICA RM 2135 microscope subjected to routine hematoxylin and eosin (H and E) staining. The slides were then examined by light microscopy. Lung, stomach, esophagus, and testis proliferate lesions were classified into cell hyperplasia, adenomas, and carcinomas. The fresh tissues were stored at -70°c. Genomic DNA was extracted by phenol-chloroform. It was obtained by proteinase K (20 mg/ml), digestion of samples in a lysis buffer (pH 7.5 EDTA 10 mM, NaCl 100 mM, Tris-HCl 100mM) and phenol-chloroform extraction.[11] One hundred nanogram of genomic DNA was mixed in polymerase chain reaction (PCR) buffer (10mM Tris-Hcl, pH 8.4, 50 mM KCl, and 1.5 mM $MgCl^2$) with 200 mM dNTP each, 0.2 µM of each 3' and 5' primer and 2.5 U AmpliTaq DNA polymerase (Boehringer, Mannheim, Germany). To amplify a 181 bp product of K-ras (exon 1), FPK primer 5'-TGATAATCTTGTGTGAGACA-3' and RPK primer 5`-CTCTATCGTAGGGTCGTACT-3` were used. The samples were amplified by PCR for 50 cycles. Each cycle consisted of 30 seconds (s) denaturation at 95°c, 30s annealing at 55°c, and 30s extension at 72°c. The final extension step was performed at 72°c for 7 minutes.[8] A negative control (1 µl of double-distilled water) was included in each PCR to exclude the possibility of PCR contamination. Amplified products were subjected to electrophoresis in 2% agarose gels and were visualized with ethidium bromide. The PCR products were purified and then sequenced. Sequencing primers were the same as described for PCR reactions.

The data on the incidence of proliferative lesions were analyzed by Fisher's exact test. Inter group differences were compared between the 3 groups. The mean difference was significant at the 0.05 level. All the statistical analysis were performed using a statistical software package (SPSS, version 11.5)

Results. One mouse belonging to the control group (C) died during the study. In the treated group, 3 mice died. No mortality was observed in the carcinogen group. There was no proliferative lesion in the dead mice. The lung proliferative lesions were observed in U and U+D

groups, but there was no proliferative lesion in the C group. They were multi focal nodular lesions, firm, white to grayish nodules located under the pulmonary pleura and often elevated slightly above the surface of the lung, their sizes were approximately 1 mm in diameter. Regarding histopathological diagnosis, there was no alveolar/bronchiolar hyperplasia alteration in any groups. In alveolar/bronchiolar adenomas showing papillary growth, glandular patterns were characteristic. They were differentiated and relatively uniform and characterized as benign tumors. Tumor cells were in the surrounding parenchyma and compression of the fibrovascular tissue (**Figures 1 & 2**). Carcinomas were characterized by invasive growth with increased mitoses, most of which were in the metaphase stage. They had glassy ground appearance with vesiculated nuclei. The cells were not uniform, with round to oval nuclei. They were composed of cuboidal to columnar epithelial cells. There were inflammatory mononuclear cell patterns overlying a delicate fibrovascular stroma (**Figure 3**). There were 1-3 cancerous lesions in all the sections. The size of each section was 1 x 1 cm^2. The alveolar/bronchiolar carcinomas demonstrated a mixed cellular pattern composed of papillary and glandular patterns. Papillary growth grew on a fine fibrovascular stroma, and those showing glandular patterns were evident as nest or as acini. The solid tumors were characterized by solid growth of more cuboidal or rounded cells. Pleomorphic and anaplastic spindle-shaped cells were also observed in these tumors. The alveolar/bronchiolar adenomas were observed in the carcinogen and treated groups, and carcinomas were observed only in the carcinogen group. In the urethane group, 30% of the animals did not develop lung tumors, 30% showed adenomas, and 40% showed adenocarcinomas. Whereas there was no proliferative lesion in 71.4% of the U+D group, 28.6% of remaining mice had adenomas in this group. The formation of lung tumor was, significantly increased in the urethane group (7 tumors) as compared with control group (no tumors) (p<0.005), but such a difference was not found in the treatment group (2 tumors) as compared with the control group. The results of incidence of

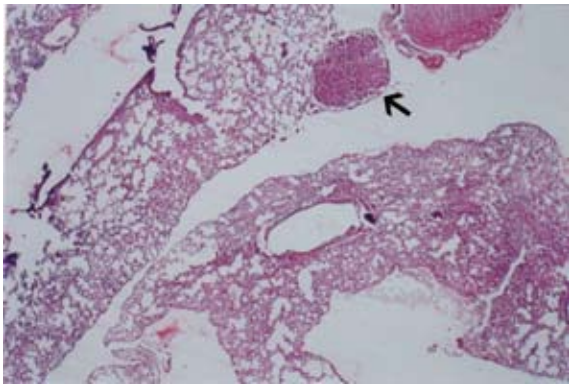

Figure 1 - An induced alveolar tumor mass in lung tissue of the urethane group.

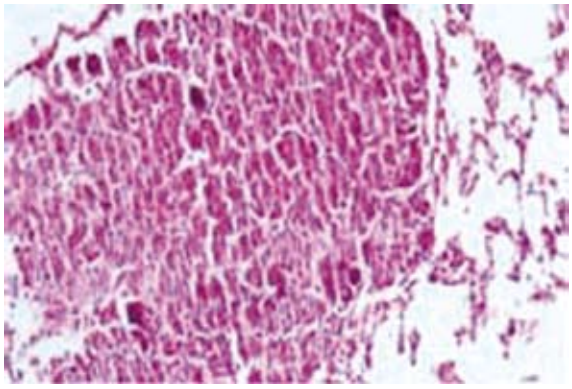

Figure 2 - Papillary adenoma in lung tissue of urethane + vitamin D group.

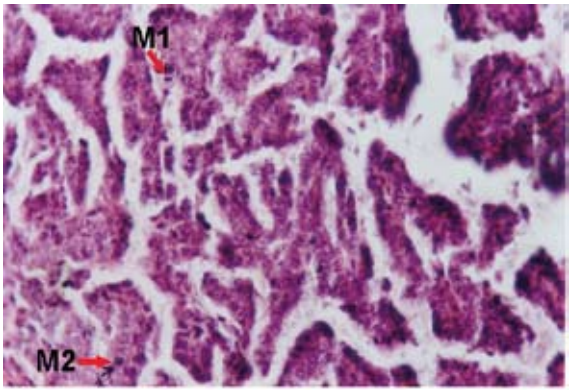

Figure 3 - Papillary adenocarcinoma in lung tissue of urethane group metaphase mitotic figure (M1), and prophase mitotic figure (M2).

Table 1 - Incidence of stomach proliferative lesions and metaplasia in the stomach of different groups.

Group	Metaplasia		Proliferative lesions		No. of animals examined
	Squamous metaplasia	Normal	Hyperplasia	Normal	
Normal control (C)	0	9	0	9	9
Carcinogen group (U)	1	9	2	8	10
Treatment group (U + D)	0	7	0	7	7

stomach proliferative lesions and metaplasia are shown in **Table 1**. There was no significant difference between all groups in stomach, esophagus and testis tissues. There was no mutation in the exon 1 of the K-ras gene of lung and stomach tissues of mice that had lung tumors and stomach metaplasias.

Discussion. Although some epidemiologic studies support a rule of sunlight, dietary vitamin D, and circulating vitamin D metabolites in reducing the risk of breast, colon, and prostate cancers, the data are mixed, and these relationships require clarification.[12] In our study, we focused on the influence of vitamin D3 on the tumors induced by urethane. Our results, confirm those of other studies,[7,9,13-17] which demonstrated urethane induced tumor in the lung tissue. Urethane-induced lesions may bypass the hyperplasia stage, thus accelerating the progression to adenoma and carcinoma and our results confirm those of another study.[6] Although according to our results urethane is tumorigenic in lung tissue, mutation in the exon 1 of the K-ras is not one of the main causative factors for the induction of tumors by urethane. Our result, confirmed those of other studies in which a role for the mutation in exon 1 of K-ras gene in lung tumors induced by urethane was excluded.[7-9] However, some studies have shown a positive role for the mutation in exon 1 of the K-ras gene in the induction of lung tumor by urethane.[6,10] The reason for the difference between our results and these studies maybe related to the kind of mice, their age, the status of being fetus or adult when exposed to urethane, frequency of exposure, and dosage of urethane. The size of tumors reflects their growth rate and is influenced by one or more pulmonary adenoma progression genes that are distinct from tumor susceptibility genes. After a few months, some of these adenomas display the nuclear atypia and invasiveness of adenocarcinomas in situ. Mice die of respiratory distress when many large adenocarcinomas develop approximately one year after carcinogen application due to a defective functioning parenchyma. Adenomas grow in a solid pattern along the alveolar septa without compressing it, or as papillary finger-like projection around a blood vessel; "mixed" tumors display both solid and papillary features. The proportion of papillary tumors increases with time after carcinogen administration, and these tumors are more aggressive and likely to progress to malignancy than solid tumors,[5] as our results suggest in the urethane group.

In our study, the formation of lung tumor was significantly increased in the urethane group as compared with the control group, however, such a difference was not found in the treatment and control groups. Our results show that vitamin D3 decreases the tumorigenic effect of urethane. Previous studies have shown that 1,25(OH)2D3 and its analogs are able to reduce the invasiveness of metastatic cancer cells in vitro. Several studies have focused on the influence of vitamin D3 and its analogs on the metastasis of lung cancer in a defined animal model.[2,3] However, there is no available study on the anti-tumorigenic effect of vitamin D3 in lung tumors induced by urethane. Our results showed an anti-tumorigenic effect of vitamin D3 in lung tumors induced by urethane in vivo. Vitamin D may reduce the risks of a tumorigenic of diet that includes high fermented foods and beverages that produce urethane in their process. The possible mechanisms by which vitamin D protects urethane-induced lung tumors may be related to the anti-proliferative effects of 1,25(OH)2D3, through down-regulation of parathyroid hormone-related protein (PTHrP) expression.[18] Also, its effects are associated with an increase in growth arrest at G0/G1 through the cycle cell phase, induction of apoptosis and differentiation, and modulation of expression of growth factor receptors. Calcitriol has the antitumor effects against many cytotoxic agents and inhibits motility and invasiveness of tumor cells and formation of new blood vessels.[19]

In our study there were only 2 cases of hyperplasia and one case of squamous metaplasia in the urethane group with no significant adenomas, carcinomas, and atypical hyperplasia of the glandular stomach being induced. Another study has shown an increased glandular stomach in the offspring of male mice exposed to urethane.[20] Stomach proliferative lesions are rare in mice; an incidence of 0.1% was reported in a 2-year study of male CD-1 mice.[21] Generation of gastric cancer normally takes a long time. Therefore, the identification of preneoplastic lesions can be a priority in order to facilitate investigation of gastric carcinogenesis. Another investigation indicated that a vitamin D3 derivative exerts chemopreventive effects against stomach carcinogenesis in rats.[22] However, in the present study, it may be due to the type of carcinogen (urethane) or the short time of study that there was no significant tumorigenic effect on the stomach, and the effect of vitamin D3 could not be examined.

In our study urethane was not found to be tumorigenic in the esophagus and we could not examine the effect of vitamin D3 as a protective factor. In contrast, previous studies had demonstrated vitamin D as a protective factor in esophageal cancer.[23,24] Notably, in the present study there was no lesion in the esophagus. Our study did not reveal any effects of urethane and vitamin D on testis tissue. Our results confirm the results of a study on urethane effect on testis tissue.[21] In another study, urethane administered in drinking water induced seminiferous tubule degeneration in mice.[16] It appears

that urethane has a carcinogenic effect in the lung tissue of mice, but not in the stomach, esophagus, and testis tissues. We surveyed only a mutation in the exon 1 of the K-ras gene, but in our study it was not one of the main factors of tumors induced by urethane. We suggest to survey mutations in both exons 1 and 2 of the K-ras gene in future studies.

Our results showed the anti-tumorigenic effect of vitamin D3 in lung tumors induced by urethane in mice. This needs to be confirmed in future studies. Longer duration of study should be carried out for investigation of urethane as a gastric carcinogen, as the generation of gastric cancer usually takes a long time.

Acknowledgment. *This work was supported by a grant from the Tabriz University of Medical Sciences. We thank Dr. Saeid Hosseini Asl and Mr. Ehsan Alvandi for their technical assistance.*

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