کارگاه‌های آموزشی مرکز اطلاعات علمی

مقاله نویسی علوم انسانی

اصول تنظیم قراردادها

آموزش مهارت های کاربردی در تدوین و چاپ مقاله
Hepatoprotective effect of silymarin in individuals chronically exposed to hydrogen sulfide; modulating influence of TNF-α cytokine genetic polymorphism

Ali Mandegary1*, Arastoo Saeedi2, Aziz Eftekhari1, Vahideh Montazeri1 and Elham Sharif3

Abstract

Background and the purpose of the study: Silymarin, a standardized extract of the milk thistle (Silybum marianum), is believed to exert some of its hepatoprotective effects though inhibition of free radicals and inflammation. In this study the effect of some pro- and anti-inflammatory cytokines and also antioxidant genes polymorphisms on the hepatoprotective effects of silymarin in the occupationally exposed individuals to hydrogen sulfide (H2S) in the sour natural gas refinery was investigated.

Methods: We genotyped seven polymorphisms in six genes reported by others as modifiers of oxidative stress (NQO1, mEPXH1, GSTT1 and GSTM1) and inflammation (TNF-α and TGF-β1) for an association in effect of decreasing in liver function tests (LFTs). The LFTs of 77 sour gas refinery workers were measured before and after administration of silymarin (140 mg, three times per day for 1 month).

Results: A significant reduction of blood AST, ALT and ALP was observed after 30 days of consumption (p < 0.001). The decreasing effect of silymarin on ALT in the subjects with high producer genotype (A allele carriers) was less than low producers. There were no significant associations between TGF-β1 and the studied genes of oxidative stress pathway and the effectiveness of silymarin.

Conclusion: This is the first report about the effectiveness of silymarin in the subjects exposed chronically to H2S. Meanwhile, the modulatory effect of TNF-α on the effectiveness of silymarin might be used for individualize therapy.

Keywords: Silymarin, Hepatoprotective, Hydrogen sulfide, Occupational toxicology, Polymorphism

Introduction

The workers of natural gas refineries are exposed to potentially toxic substances like hydrogen sulfide (H2S) and benzene. Recently we have shown that the oxidative stress biomarkers increased in the workers of sweet and, in higher levels, sour gas refineries [1]. Meanwhile the levels of liver function tests (LFTs) were high in the H2S exposed subjects [1]. Although pathogenesis of liver toxicity induced by H2S has not been elucidated so far, there is experimental evidence that induction of oxidative stress plays a key role [2,3]. Several observations suggest that increased reactive oxygen species (ROS) and resulting oxidative stress is a critically important determinant of liver injury [4,5]. Oxidative stress is involved in the release of pro-inflammatory mediators (cytokines and chemokines) and injury to the liver cells [4,5]. Silymarin, a mixture of flavonolignans isolated from Silybum marianum (milk thistle), has been used to treat liver diseases for hundreds of years [6,7]. The hepatoprotective effect of silymarin is mainly due to its strong antioxidant activity and scavenging the free radicals [8]. It also effectively inhibits the inflammation [9,10], which can also damage the liver [6,11]. Silybin is an effective antioxidant, conserving GSH in liver cells while stabilizing the liver cell membranes against oxidative attack [4,12].
There are inter-individual variations in detoxifying enzymes and cytokines that could be translated into differences in susceptibility for xenobiotic toxicity and treatment outcomes [13-17]. So in this study, we decided to investigate the association of polymorphisms in the enzymes involved in general oxidative stress defence including NAD(P)H:quinone oxidoreductase-1 (NQO1), Glutathione S-transferases (GST) M1 and T1, and microsomal epoxide hydrolase (EPHX1) enzymes and the two most important cytokines involved in the pathogenesis of liver disease with the basis of inflammation, namely tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) cytokines and the treatment outcome of silymarin in the subjects chronically exposed to H₂S.

Materials and methods

Materials

Silymarin containing tablets (Livergol) was purchased from Goldaru, Isfahan, Iran. PCR master mix was obtained from CinnaClone (Iran). Ethylenediamine tetraacetic acid (EDTA), Tris, agarose, Sodium Dodecyl Sulfate (SDS), isopropanol and ethanol were purchased from Merck (Germany). Proteinase K and DNA ladder were obtained from Fermentas (Germany).

Study subjects

In this cross-sectional study, 77 employees of sour gas refinery were recruited. According to local authorities, the atmospheric concentration of H₂S in the place was 10–15 ppm [1]. The eligibility criteria for the cases were as follow: working at least 5 years in the refinery and no history of liver disease. Demographic data, general health conditions, lifestyle, years of employment and smoking habits were registered in a questionnaire. Written informed consent was obtained from the subjects after describing the aim of the study. The study was conducted according to the declaration of Helsinki and subsequent revisions and approved by the Ethical Committee of Kerman University of Medical Sciences (No: 91/91/k, KMU). The liver function tests (LFTs) including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined before and after administration of silymarin at the dosage of 150 mg three times per day (Livergol, Goldaru, Esfahan, Iran).

DNA extraction and GST genotyping

DNA was extracted from 5 ml peripheral blood using Miller method [18]. The subjects were genotyped for the deletion in GSTM1 and GSTT1 by a multiplex polymerase chain reaction (PCR). The PCR conditions and primers were as described previously [19]. TNF-α -308G/A and TGF-β1 codon 10 and codon 25 genotyping methods were based on the PCR-ARMS, and were similar to those previously described [20]. The amplified fragments were 184-bp, 241-bp and 233-bp respectively for TNF-α, TGF-β1 codon 10 and 25. EPXH1 exon 4 (His139Arg), NQO1 (C609T) and TNF-α (G-308A, for confirmation of PCR-ARMS for TNF-α -308G/A) genotyping methods were based on PCR-RFLP and as described before [21-23]. The products of each PCR reaction and digestion were resolved by electrophoresis on 3% agarose gels stained with ethidium bromide. Reliability and validity of the PCR and RFLP methods were assessed through re-conducting the genotype assays using at least a 10% sample of our DNA samples. The polymorphisms of heterozygous, homozygous and wild-type TNF-α which obtained by PCR-ARMS were re-checked with PCR-RFLP method. In addition, for TGF-β1 the method was also assessed through re-conducting the genotype assays using most of DNA samples with mutations (both heterozygous and homozygous). The results for all re-assessments were 100% concordant.

Statistical analysis

For the comparison of continuous variables, we firstly checked the assumption that they were normally distributed. If the distribution was normal, the results were expressed as means and standard error (SE) and a classical t-test or one-way ANOVA was used accordingly. According to the genes polymorphisms, genotypes were collapsed into two categories: homozygous (common genotypes) versus heterozygous + variant homozygous and they were coded as follow: GSTM1*0 (code 0) and GSTM1-positive (code 1), GSTT1*0 (code 0) and GSTT1-positive (code 1), NQO1 CC allele (code 0) and CT/TT alleles (code 1), EPXH exon 4 AA allele (code 0) and AG/GG alleles (code 1), TGF-β1 codon 10 TT allele (code 0, high producer) and all other alleles (code 1, low producer) and TGF-β1 codon 25 GG allele (code 0, high producer) and GC/CC alleles (code 1, low producer). Combination of TGF-β1 codons 10 and 25 was as follow: TT/CC (code 0, high producer) and all other combinations (code 1, low producer). Simple and multiple linear regression was used to screen the independent variables including genetic polymorphism to best predict the value of the LFTs and LFTs difference. Age and BMI variables believed to potentially confound the association with LFTs’ level were included in the multivariate model. The significance (P) of genetic polymorphisms factors from multivariate analysis was considered as adjusted p-value. Differences between LFTs before and after administration of silymarin were analyzed using paired-sample t-test. For all the tests, a p-value less than 0.05 was considered to be significant. All analyses were conducted using SPSS statistical software (version 11.5).

Results

Significant decreases in AST (P < 0.01), ALT (P < 0.001) and ALP (P < 0.001) were observed by use of silymarin
The mean ± SE of AST before and after using were 39.8 ± 0.78 and 29.7 ± 0.07 U L⁻¹ (Figure 1A). A significant (P < 0.001) decrease in ALT was observed after administration of silymarin (66.4 ± 1.4 U L⁻¹ before versus 30.5 ± 0.9 U L⁻¹ after (Figure 1B). After use of silymarin, the ALP level decreased significantly (P < 0.001). The mean ± SE values before and after were 231.2 ± 5.9 and 209.2 ± 4.8 U L⁻¹ (Figure 1C).

Simple and multivariate linear regression were performed to analyze the association of GSTM1, GSTT1, NQO1 and EPXH1 genes polymorphisms with changes in LFTs after administration of silymarin (Table 1). There was no association between the abovementioned genes’ polymorphisms and decreasing in LFTs after administration of silymarin.

Simple and multivariate linear regression were performed to analyze the association of TNF-α, TGF-β1 codon 10, TGF-β1 codon 25 and combination of these two codons polymorphisms with changes in LFTs after administration of silymarin (Table 2). TNF-α G-308A polymorphism was significantly associated with decreasing in the AST (p = 0.04) and ALT (p = 0.003). These results show that decreasing in the AST and ALT levels are lower in the subjects with high producer TNF-α genotype. These associations remained significant after adjustment with age and BMI. There was also negative significant association between TGF-β1 codon 10 polymorphisms and decreasing in ALP that this association was abolished when age and BMI added to the model.

**Discussion**

In light of our recent study demonstrating increase in the LFT levels in the workers exposed chronically to H₂S, we became interested in determining whether a well-known hepatoprotective natural product like silymarin could decrease the LFTs in the subjects and if the genetics of the subjects could determine responding to treatment. Administration of silymarin (140 mg tablets, thrice per day for 1-month) significantly reduced the LFTs in the subjects. The hepatoprotective mechanisms of silymarin on H₂S-induced elevation of LFTs have not been addressed so far. Recently we have also shown that stress oxidative biomarkers were higher in the workers in exposure to H₂S [1]. Lines of evidence demonstrate that stress oxidative is one of the main mechanisms for the toxic effect of xenobiotics on the liver [4,15,24]. Antioxidant activity and scavenging the free radicals have been reported as the main mechanism for the hepatoprotective effect of silymarin [6].

Our data showed significant association (B = -8.30, CI: -13.73,-2.86) between the polymorphism of TNF-α G-308A and the changes in ALT and AST after administration of silymarin (Table 2). In other words, decrease in ALT and AST in the high TNF-α producers treated with silymarin is significantly lower than low producers. TNF-α is a pro-inflammatory cytokine involved in the pathogenesis of variety of liver injuries [25]. TNF-α, in concert with other cytokines, mediates the recruitment of neutrophils to the liver that induce inflammation and cell death [26]. Production of TNF-α is under genetic control which determined by a number of single nucleotide polymorphisms in this gene. The G-308A polymorphism in the promoter of the gene is the most studied one that has been linked to higher TNF-α production [27,28] as homozygosity for
Table 1 Association of oxidative stress related genes polymorphisms with changes in LFTs’ levels by consumption of silymarin

| LFT | Gene | Unadjusted B (CI) | p-value | Adjusted B (CI) | p-value |
|-----|------|------------------|---------|----------------|---------|
| AST | NQO1 | −0.73 (−3.38, 4.84) | 0.72 | −0.05 (−4.10, 3.92) | 0.98 |
|     | GSTM1| −0.58 (−4.02, 3.03) | 0.75 | −0.64 (−4.25, 2.97) | 0.72 |
|     | GSTT1| 1.27 (−3.10, 5.64)  | 0.56 | 1.92 (−2.55, 6.38) | 0.40 |
|     | EPXH4| 1.34 (−3.10, 5.77)  | 0.54 | 1.72 (−2.73, 6.17) | 0.44 |
| ALT | NQO1 | 0.87 (−4.20, 5.92)  | 0.73 | 0.79 (−4.40, 5.98) | 0.76 |
|     | GSTM1| −4.24 (−8.62, 0.13) | 0.05 | 4.21 (−8.67, 0.25) | 0.06 |
|     | GSTT1| 1.30 (−4.23, 6.83)  | 0.64 | 1.22 (−4.50, 6.92) | 0.65 |
|     | EPXH4| 1.04 (−3.02, 4.67)  | 0.54 | 0.89 (−4.46, 6.24) | 0.74 |
| ALP | NQO1 | −12.83 (−39.89, 14.22) | 0.35 | −2.64 (−20.22, 14.95) | 0.76 |
|     | GSTM1| 1.21 (−23.79, 26.21) | 0.92 | 2.27 (−13.67, 18.22) | 0.77 |
|     | GSTT1| 21.28 (−8.93, 51.50) | 0.16 | 10.21 (−9.46, 29.87) | 0.30 |
|     | EPXH4| −14.20 (−47.62, 19.22) | 0.40 | 1.73 (−17.78, 21.24) | 0.86 |

Multivariate regression: Parameters adjusted for age and BMI; B: Partial regression coefficient.

Table 2 Association of inflammation related cytokines polymorphisms with changes in LFTs’ levels by consumption of silymarin

| LFT | Gene          | Unadjusted B (CI) | p-value | Adjusted B (CI) | p-value |
|-----|---------------|------------------|---------|----------------|---------|
| AST | TNF-α         | −4.27 (−8.57, 0.03) | 0.05 | −4.58 (−8.97, −0.21) | 0.04* |
|     | TNF-β1 codon 10 | −0.77 (−5.17, 3.61) | 0.72 | −0.07 (−4.56, 4.41) | 0.97 |
|     | TNF-β1 codon 25 | 2.78 (−3.36, 8.93)  | 0.36 | 2.79 (−3.40, 8.98)  | 0.37 |
|     | TNF-β1 codons 10/25 | 0.99 (−3.04, 5.03) | 0.62 | 1.37 (−2.73, 5.46) | 0.50 |
| ALT | TNF-α         | −8.30 (−13.73, −2.86) | 0.003* | −8.93 (−14.62, −3.23) | 0.003* |
|     | TNF-β1 codon 10 | −3.62 (−8.92, 1.67)  | 0.17 | −3.43 (−9.00, 2.14)  | 0.22 |
|     | TNF-β1 codon 25 | 5.87 (−1.29, 13.03) | 0.10 | 5.94 (−1.36, 13.24) | 0.10 |
|     | TNF-β1 codons 10/25 | −0.33 (−5.28, 4.63) | 0.89 | 0.03 (−5.11, 5.24) | 0.99 |
| ALP | TNF-α         | −6.17 (−41.08, 28.75) | 0.72 | −0.85 (−21.93, 20.21) | 0.93 |
|     | TNF-β1 codon 10 | −31.45 (−61.31, −1.59) | 0.03 | −9.05 (−27.43, 8.32) | 0.33 |
|     | TNF-β1 codon 25 | 16.67 (−25.38, 58.73) | 0.43 | −12.00 (−37.17, 13.17) | 0.34 |
|     | TNF-β1 codons 10/25 | −16.16 (−44.41, 12.07) | 0.25 | −8.65 (−24.63, 7.33) | 0.28 |

Multivariate regression: Parameters adjusted for age and BMI. *Significant effect. B: Partial regression coefficient.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AM participated in the design of the study, performed the statistical analysis, participated in the sequence alignment and drafted the manuscript. AS carried out sampling and carried out genotyping of some genes. AE carried out genotyping of some genes and filed the data. VM carried out genotyping of some genes. ESh carried out DNA extraction and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This work is supported by Deputy of Research, Kerman University of Medical Sciences. The authors would like to thank Ms. Maryam Sezavar and Dr. Samaneh Soleymani for their helps. This article also has been derived from the thesis of MSc students (AS and AE) in Kerman University of medical Sciences, Faculty of Pharmacy, Kerman, Iran.

Author details
1 Department of Pharmacology & Toxicology, School of Pharmacy and Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran. 2 Physiology Research Center, Kerman University of Medical Sciences, Kerman, Iran. 3 Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran.

Received: 20 October 2012 Accepted: 17 March 2013

Published: 8 April 2013

References
1. Mandegary A, Sezavar M, Saeedi A, Amirheidari B, Naghibi B: Oxidative stress induced in the workers of natural gas refineries, no role for GSTM1 and GSTT1 polymorphisms. Hum Exp Toxicol 2012, 31:1271–1279.
2. Eghbal MA, Pennefather PS, O’Brien PJ: H2S cytotoxicity mechanism involves reactive oxygen species formation and mitochondrial depolarisation. Toxicology 2004, 203:69–76.
3. Attane-Ramos MS, Wagner ED, Gakens HR, Plewa MJ: Hydrogen sulfide induces direct radical-associated DNA damage. Mol Cancer Res 2007, 5:455–459.
4. Ha HL, Shin HJ, Feitelson MA, Yu DY: Oxidative stress and antioxidants in hepatic pathogenesis. World J Gastroenterol 2010, 16:6035–6043.
5. Jaeschke H: Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol 2011, 26(Suppl 1):173–179.
6. Pradhan SC, Girish C: Reactive oxygen species and its role in hepatic pathogenesis. Indian J Med Res 2006, 124:491–504.
7. Saller R, Brigolin R, Melzer J, Meier R: An updated systematic review with meta-analysis for the clinical evidence of silymarin. Forsch Komplementmed 2008, 15:39–50.
8. Tsai JH, Liu JY, Wu TT, Ho PC, Huang CY, Shyu JC, Hsieh YS, Tsai CC, Liu YC: Silybum marianum extracts prevent liver damage. World J Hepatol 2010, 2:176–182.
9. De La Puerta R, Martinez E, Bravo L, Ahumada MC: Effect of silymarin on liver fibrosis induced by carbon tetrachloride in rats. J Viral Hepat 2008, 15:508–514.
10. De La Puerta R, Martínez E, Bravo L, Ahumada MC: Effect of silymarin on different acute inflammation models and on leukocyte migration. J Pharm Pharmacol 1996, 48:968–970.
11. Kalijar SK: Silymarin and skin cancer prevention: anti-inflammatory, antioxidant and immunomodulatory effects (Review). Int J Oncol 2005, 26:169–176.
12. Luster MI, Sirineanova PP, Gallucci RM, Brucolieri A, Blazka ME, Vyousev B: Role of inflammation in chemical-induced hepatotoxicity. Toxicol Lett 2001, 120:317–321.
13. Shaker E, Mahmoud H, Mnaa S: Silymarin, the antioxidant component and Silibum marianum extracts prevent liver damage. Food Chem Toxicol 2010, 48:803–806.
14. Labdi J, Keen L, Gallagher G, Kimberly R, Huizinga T, McDermott MF, Oksenberg J, McMillil J, Pociot F, Hardt C, D’Alfonso S: Cytokine gene polymorphism in human disease: on-line databases. Genes Immun 1999, 1:3–19.
کارگاه‌های آموزشی مرکز اطلاعات علمی

مقاله نویسی علوم انسانی

اصول تنظیم قراردادها

آموزش مهارت های کاربردی در تدوین و چاپ مقاله