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Original Article

Brief Report

Case Report

CONTENTS (*Continued*)

Original Article

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The potential antifibrotic impact of apocynin and alpha-lipoic acid in concanavalin A-induced liver fibrosis in rats: Role of NADPH oxidases 1 and 4

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Summary **Liver fibrosis results from chronic inflammation that precipitates excessive accumulation of extracellular matrix. Oxidative stress is involved in its pathogenesis. This study aimed to elucidate the potential antifibrotic effect of the NADPH oxidase (NOX) inhibitor, apocynin against concanavalin A (ConA)-induced immunological model of liver fibrosis, and to investigate the ability of the antioxidant, alpha-lipoic acid (α-LA) to potentiate this effect. Rats were treated with apocynin and/or α-LA for six weeks. Hepatotoxicity indices, oxidative stress, insulin, NOXs, inflammatory and liver fibrosis markers were assessed. Treatment of animals with apocynin and α-LA significantly ameliorated the changes in liver functions and histopathological architecture induced by ConA. Liver fibrosis induced by ConA was evident where alpha-smooth muscle actin and transforming growth factor-beta1 were elevated, which was further confirmed by Masson's trichrome stain and increased hydroxyproline. Co-treatment with apocynin and α-LA significantly reduced their expression. Besides, apocynin and α-LA significantly ameliorated oxidative stress injury evoked by ConA, as evidenced by enhancing reduced glutathione content, antioxidant enzymes activities and decreasing lipid peroxides. ConA induced a significant elevation in serum insulin level and inflammatory markers; tumor necrosis factor-alpha, interleukin-6 and nuclear factor kappa b. Furthermore, the mRNA tissue expression of NOXs 1 and 4 was found to be elevated in the ConA group. All these elevations were significantly reduced by apocynin and α-LA co-treatment. These findings indicate that using apocynin and α-LA in combination possess marked antifibrotic effects, and that NOX enzymes are partially involved in the pathogenesis of ConA-induced liver fibrosis.**

Keywords: Concanavalin A, liver fibrosis, apocynin, alpha-lipoic acid, NADPH oxidase, NOX inhibitor

1. Introduction

Liver fibrosis results from chronic damage to the liver and accumulation of extracellular matrix (ECM) proteins, which is a characteristic of most types of chronic liver diseases (*1*). There is compelling evidence of hepatic cellular recovery with possible remodeling of scar tissue (*2*). Oxidative stress has been identified as a key mechanism of fibrogenesis. Upon activation of kupffer cells, they secrete inflammatory and fibrogenic mediators. These mediators along with reactive oxygen species (ROS) activates hepatic stellate cells (HSCs) (*3*). NADPH oxidase (NOX) enzymes is indeed a main source of oxidative stress in hepatocytes and non-hepatocytes (*4*). They consist of seven transmembrane proteins (NOXs 1 to 5 and Duox 1 and 2), whose primary function is to catalyze the transfer of electrons

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from NADPH to O_2 generating superoxide and hydrogen peroxide (*5*). Evidence indicates a crucial role for NOX-mediated ROS generation in hepatic fibrogenesis (*6,7*).

Apocynin, isolated from *Picrorhiza kurroa* extracts, is a NOX inhibitor that was shown to interfere with membrane assembly of cytosolic subunits of the NOX complex. Apocynin reduced the expression of $gp91^{phox}$ (a NOX subunit), where it replenished cellular NADPH leading to alleviated hepatic oxidative injury, which may underlie its therapeutic potency (*8*). Besides, alpha-lipoic acid (α-LA) was shown to possess a beneficial role in chronic liver diseases. This is mediated *via* its anti-inflammatory and antioxidant activities that inhibit the activation of HSCs (*9*). Moreover, administration of apocynin markedly enhanced the neuroprotection effect of α-LA in a rat model of ischemia/reperfusion injury, a finding that may reflect the promising beneficial effect of using the two drugs together (*10*).

Accordingly, the present study was attempted to provide an update on the hepatoprotective effects and the undisclosed antifibrotic mechanisms of apocynin, α-LA and their combination against concanavalin A (ConA)-induced liver fibrosis in rats. This model is considered as a murine model of autoimmune hepatitis (T-cell mediated) that resembles the pathological changes accompanying autoimmune and viral hepatitis in human. Also, this study aimed to assess the potential role that NOX-1 and NOX-4 might play in the pathogenesis of ConA-induced liver injury.

2. Materials and Methods

2.1. *Drugs and chemicals*

ConA, apocynin and α-LA were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and formaldehyde (37%) were purchased from El-Gomhouria Chemical Co. (Cairo, Egypt). All other chemicals and solvents were of highest grade commercially available.

2.2. *Animals*

The study protocol was conducted according to the ethical guidelines (Faculty of Pharmacy, Ain Shams University, Egypt). Male Wistar rats (150-200 g) were obtained from Nile Co. for Pharmaceutical and Chemical Industries, Egypt. Rats were housed in an air-conditioned atmosphere, at a temperature of 25°C with alternatively 12 h light and dark cycles. Animals were acclimated for 2 weeks before experimentation and kept on a standard diet and water *ad libitum*.

2.3. *Experimental design*

Rats were divided into 7 groups (n = 15) and treated for 6 consecutive weeks. Group 1 received phosphate buffered saline (PBS) (once/week, *i.v.*) and DMSO (3 times/week, *i.p.*). Groups 2 and 3 received only apocynin or α-LA, respectively (50 mg/kg, dissolved in DMSO, 3 times/week, *i.p.*). The doses of apocynin and α-LA were chosen as a result of a pilot study in our lab. Group 4 received ConA (20 mg/kg, dissolved in PBS, once/week, i.v.). Group 5, 6 and 7 received ConA together with apocynin, α-LA or both, respectively.

At the end of the 6 weeks, blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 1,000 g for 10 min and used for the assessment of liver functions and insulin level. Then, rats were sacrificed, and liver tissues were dissected, weighed and washed with ice-cold saline. Specimens from the three major lobes of each liver from the different treatment groups were fixed in formalin 10% for histopathological examination and detection of fibrosis markers; Masson's trichrome staining, alpha-smooth muscle actin (α-SMA) and transforming growth factor-beta1 (TGF-β1). Other liver specimens were homogenized in ice-cold saline and the homogenate was used to assess oxidative stress markers; reduced glutathione (GSH) and lipid peroxides (MDA), antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT), inflammatory makers; nuclear factor kappa b (NF-κB), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and mRNA expression of NOX-1 and NOX-4.

2.3.1. *Assessment of hepatotoxicity indices*

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method of Reitman and Frankel (*11*). Serum levels of total bilirubin, total cholesterol (TC), triglycerides (TG) and albumin were estimated using available commercial kits (Spectrum diagnostics, Cairo, Egypt). Liver index was calculated according to the formula: (liver weight/body weight) × 100.

2.3.2. *Histopathological examination*

Autopsy samples were fixed in 10% forlmalin for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain (*12*).

2.3.3. *Assessment of oxidative stress markers and antioxidant enzymes*

GSH and MDA content were determined according to the methods of Ellman (*13*) and Mihara and Uchiyama (*14*), respectively. The activities of SOD and CAT were determined using the methods of Nishikimi, Appaji Rao (*15*) and Aebi (*16*), respectively.

2.3.4. *Assessment of inflammatory markers and insulin*

Determination of IL-6, TNF-α, NF-κB and insulin levels were performed using commercial rat ELISA kits (Immuno-Biological Laboratories, Minnesota, USA), (RayBiotech, Inc., Norcross, Georgia, USA), (Cloud-Clone Corp., Texas, USA) and (Société de Pharmacologie et d'Immunologie-BIO, Montigny-le-Bretonneux, France), respectively, according to the manufacturer's instructions.

2.3.5. *Assessment of liver fibrosis*

Liver fibrosis was evaluated using Masson's trichrome stain and by measuring the hydroxyproline content according to the method of Reddy and Enwemeka (*17*). Liver content of α-SMA and TGF-β1 were examined immunohistochemically with the following primary antibodies; mouse monoclonal to rat α-SMA (A2547, Sigma-Aldrich Chemical Co., St Louis, MO, USA) and mouse monoclonal to rat TGF-β1 (T0438, Sigma-Aldrich Chemical Co., St Louis, MO, USA). The images were then quantified by using image analysis software (Image J, 1.46a, NIH, USA), and represented as the area percentage of the immunopositive reaction per field (×400).

2.3.6. *Assessment of NOX-1 and NOX-4 mRNA expression*

NOX-1 and NOX-4 mRNA expression were estimated by quantitative real-time polymerase chain reaction (qRT-PCR). For RNA extraction, total RNA from liver tissue was extracted using the QIAzol and RNeasy mini kit (QIAGEN, California, USA), as recommended by the manufacturer. RNA samples were then reverse transcribed and processed for PCRs. The primers of NOX-1, NOX-4 and the internal control β-actin were designed and synthesized by Invitrogen, USA. qRT-PCR was performed using the Applied Biosystems 7500 RT-PCR system. PCR samples were activated at 94°C for 10 min followed 35 cycles that were performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The mRNA expression of NOX-1 and NOX-4 was calculated based on the method of $2^{-(\Delta\Delta Ct)}$, where Ct is cycle threshold. The primers were as follows: NOX-1 forward: 5′AACAACAGCACTCACCAATG3′, reverse: 5′TCAAGAAGGAAGCAAAGGG3′, NOX-4 forward: 5′TCAACTGCAGCCTGATCCTTT3′, reverse: 5′CTGTGATCCGCGAAGGTAAG3′ and β-actin forward: 5′CCCAGCACAATGAAGATCAAGATCAT3′, reverse: 5′ATCTGCTGGAAGGTGGACAGCGA3′.

2.4. *Statistical analysis*

Data are presented as mean ± S.D. Multiple comparisons were performed using one-way ANOVA followed by Tukey-Kramer as a post-hoc test, as appropriate. The 0.05 level of probability was used as the criterion for significance. All statistical analyses and graphs were performed using GraphPad Prism (ISI® software, USA) version 5 software

3. Results

3.1. *Hepatotoxicity indices*

ConA intoxication significantly increased the levels of ALT and AST by 161 and 140%, respectively, when compared to the control group. In contrast, co-treatment with both apocynin and α-LA significantly lowered the levels of ALT and AST by 59 and 52%, respectively, when compared to the ConA group. Also, ConA induced a significant decrease in albumin level by 34% and a significant increase in the levels of total bilirubin, TC, TG as well as the liver index by 269, 88 and 174 and 38%, respectively, as compared to the control group. Interestingly, the combination group showed a more pronounced increase in albumin level by 44% and a significant decrease in the serum levels of total bilirubin, TC, TG as well as the liver index accounting for 68, 43 and 56 and 27%, respectively, as compared to the ConA group (Table 1).

Table 1. Effects of treatment with 50 mg/kg apocynin and/or 50 mg/kg α-LA on liver function tests in ConA-induced liver injury in rats

Treated group	Liver index (%)	ALT (IU/L)	AST (IU/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	TC (mg/dL)	TG (mg/dl)
Control	$3.6^{b} \pm 0.29$	$27.4^{b} \pm 5.7$	$107.7^{b} \pm 17.5$	$0.22^{b} \pm 0.06$	$4.33^{b} \pm 0.63$	$57.2^{b} \pm 8.70$	$37.4^{b} \pm 14.3$
Apocynin	$3.4^{b} \pm 0.13$	$24.6^{b} \pm 6.5$	$104.2^{b} \pm 11.1$	$0.20^{b} \pm 0.06$	$4.33^{b} \pm 0.50$	$61.7^{b} \pm 21.3$	$22.8^{b} \pm 7.20$
α-LA	$3.7^{b} \pm 0.46$	$29.7^{b} \pm 4.1$	$108.7^{b} \pm 35.0$	$0.22^{b} \pm 0.05$	$4.18^{b} \pm 0.33$	81.1 ± 9.60	$24.7^{b} \pm 6.10$
ConA	$4.9^{a,c} \pm 0.29$	$71.4^{a,c} \pm 4.9$	$258.7^{a,c} \pm 60.1$	$0.83^{a,c} \pm 0.15$	$2.85^{a,c} \pm 0.56$	$107.7^{a,c} \pm 25.7$	$102.7^{a,c} \pm 23.5$
ConA + Apocynin	$4^{b} \pm 0.17$	$35.4^{b} \pm 11.2$	$159.2^{b} \pm 23.9$	$0.40^{b} \pm 0.19$	$3.98^{b} \pm 1.13$	$62.8^{b} \pm 8.70$	$37.1^{b} \pm 14.2$
ConA + α-LA	$4.2^{a,b,c} \pm 0.33$	$39.5^{b} \pm 8.0$	$164.7^{b} \pm 44.1$	$0.39^{b} \pm 0.15$	3.89 ± 0.33	$96.8^{a,c} \pm 19.5$	$44.6^{b} \pm 4.20$
ConA + Apocynin + α-LA	$3.6^{b} \pm 0.23$	$29.5^{b} \pm 7.3$	$124.4^{b} \pm 36.5$	$0.26^{b} \pm 0.07$	$4.11^{b} \pm 0.29$	$61.1^{b} \pm 7.50$	$45.6^{b} \pm 12.8$

Data are presented as means ± S.D. ($n = 15$). [a], [b] and [c]: Significantly different from control, ConA and ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglycerides.

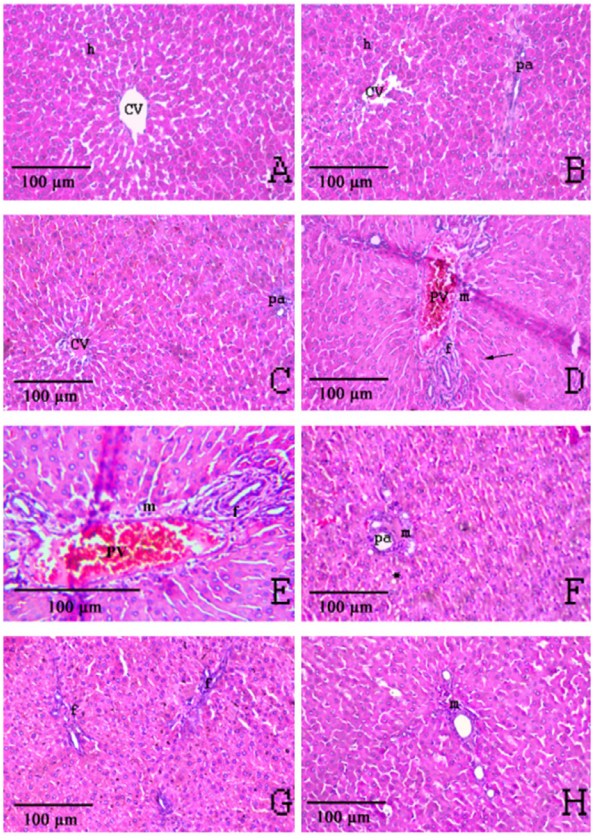

Figure 1. Representative photomicrographs of liver sections stained with haematoxylin and eosin (×400). A, B and **C:** Sections taken from the livers of rats in control, apocynin or α-LA, respectively, showing normal histological structure of the central vein (CV) and surrounding hepatocytes (h). **D:** Section taken from a liver of a rat intoxicated with ConA shows severe dilatation and congestion of the portal vein (PV) with fibrosis (f) and inflammatory cells infiltration (m) in the portal area surrounding the bile duct and diffused kupffer cells proliferation (arrow) in between the hepatocytes. **E:** Magnification at (×640) of the section of the liver shown in **D**. **F:** Section taken from the liver of a rat pretreated with apocynin showing few inflammatory cells infiltration (m) in the portal area (pa) with fatty changes (*) in few hepatocytes. **G:** Section taken from a liver of a rat pretreated with α-LA shows few fiboblastic cells (f) proliferation in the portal area. **H:** Section taken from a liver of a rat co-treated with both apocynin and α-LA showing few inflammatory cells infiltration (m) in the portal area surrounding the bile duct.

3.2. *Histopathological examination*

Liver sections from the control, apocynin or α-LA groups showed normal hepatic architecture (Figures 1A, 1B and 1C). Chronic intoxication with ConA induced fibroblastic cells proliferation with infiltrated inflammatory and kupffer cells in the portal area (Figures 1D and 1E). However, liver specimens from rats treated with ConA and either apocynin or α-LA showed improvements in the histopathological changes (Figures 1F and 1G). Interestingly, co-treatment with both apocynin and α-LA preserved the normal architecture of hepatic parenchyma (Figure 1H).

3.3. *Oxidative stress markers and antioxidant enzymes*

As expected, ConA-intoxicated rats showed significantly reduced content of GSH by 84% and elevation of MDA by 148%, as compared to the control group. Co-treatment of animals with both apocynin and α-LA produced further improvement in oxidative stress markers than any of them alone, where GSH content was significantly increased by 226% and MDA level was reduced by 38% compared to ConA group (Table 2). In addition, ConA-intoxicated group showed a significant decrease in SOD and CAT activities by 85%, as compared to the control group. Remarkably, the combination of the two drugs resulted in a significant increase in the activity of SOD by 38% with respect to the apocynin only-treated group. Furthermore, this group showed a significant increase in the activities of SOD and CAT accounting to 82 and 64%, respectively, with respect to the α-LA only-treated group (Table 2).

3.4. *Inflammatory markers*

It was found that the group injected with ConA showed about 5-fold increase in TNF-α and IL-6, as compared to the control group. In contrast, co-treatment with both apocynin and α-LA revealed significantly lowered expression of TNF-α and IL-6 by 73 and 80%,

Table 2. Effects of treatment with 50 mg/kg apocynin and/or 50 mg/kg α-LA on oxidative stress markers and antioxidant enzymes in ConA-induced liver injury in rats

Treated group	GSH (µmol/g tissue)	MDA (nmol/g tissue)	SOD (U/g tissue)	CAT (U/g tissue)
Control	$4.0^{b,c} \pm 0.2$	$54.6^{b,c} \pm 11.3$	$13.2^{b,c} \pm 0.5$	$13.5^{b,c} \pm 0.6$
Apocynin	$3.8^{b,c} \pm 0.2$	$55.3^{b,c} \pm 9.3$	$12.2^{b,c} \pm 0.8$	$12.7^{b,c} \pm 1.2$
α-LA	$3.3^{a,b,c} \pm 0.2$	$62.8^{b,c} \pm 4.5$	$9.9^{a,b,c} \pm 0.7$	$11.3^{b,c} \pm 1.7$
ConA	$0.6^{a,c} \pm 0.1$	$135.3^{a,c} \pm 15.8$	$2.0^{a,c} \pm 0.2$	$1.9^{a,c} \pm 0.1$
ConA + Apocynin	$1.5^{a,b,c} \pm 0.1$	$102.8^{a,b} \pm 12.1$	$4.2^{a,b,c} \pm 0.3$	$5.2^{a,b} \pm 0.4$
ConA + α-LA	$1.2^{a,b,c} \pm 0.1$	$120.1^{a,c} \pm 14.7$	$3.1^{a,c} \pm 0.2$	$4.1^{a,c} \pm 0.2$
ConA + Apocynin + α-LA	$2.1^{a,b} \pm 0.1$	$84.2^{a,b} \pm 8.5$	$5.8^{a,b} \pm 0.4$	$6.7^{a,b} \pm 0.4$

Data are presented as means ± S.D. ($n = 15$). [a], [b] and [c]: Significantly different from control, ConA and ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test. GSH, reduced glutathione; MDA, lipid peroxides; SOD, superoxide dismutase; CAT, catalase.

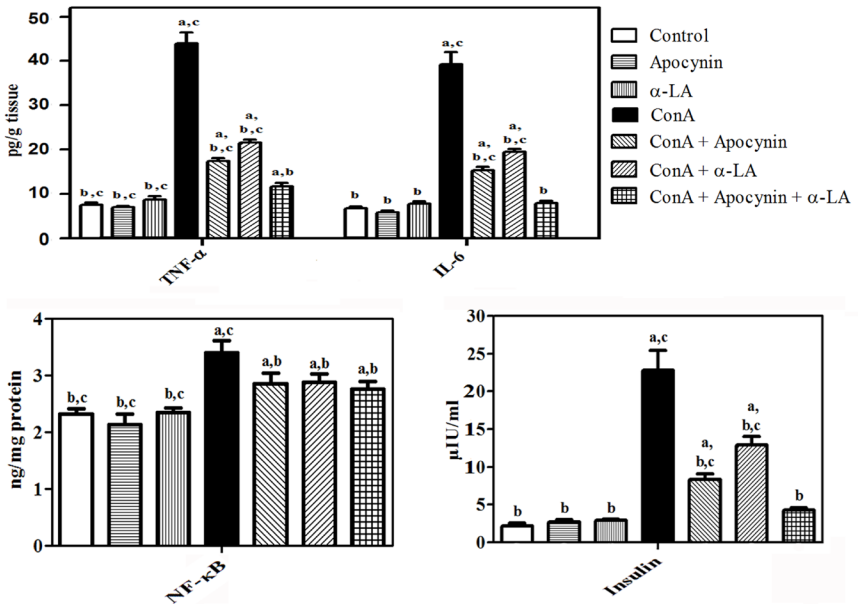

Figure 2. Effect of treatment with 50 mg/kg apocynin and/or 50 mg/kg α-LA on TNF-α, IL-6 and NF-κB tissue levels as well as serum insulin level in ConA-induced liver fibrosis in rats. Values are given as mean ± S.D. TNF-α, IL-6 and insulin (n = 15), NF-κB (n = 6). a, b or c: Significantly different from the control, ConA or ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test. TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; NF-κB, nuclear factor kappa b.

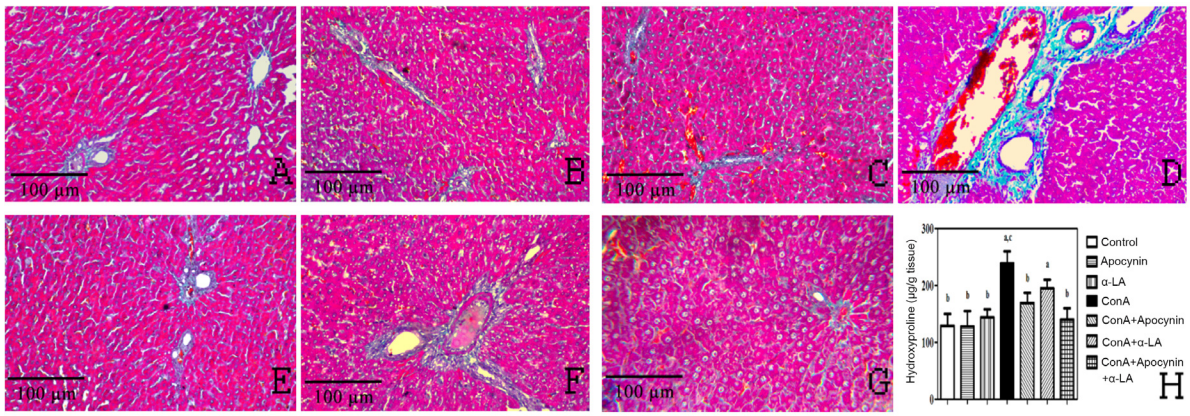

Figure 3. Representative photomicrographs of liver sections stained by Masson's trichrome (×400). A, B and **C:** Sections obtained from livers of rats in control, apocynin or α-LA, respectively, showing normal histological structure in the portal area with minimal collagen deposition. **D:** Section taken from a liver of a rat intoxicated with ConA showing severe congestion in the portal vein and excessive collagen fibers deposition along with pseudolobules formation in the portal area. **E:** Section taken from the liver of a rat pretreated with apocynin showing nearly an absence of pseudolobules with less collagen deposition. **F:** Section taken from a liver of a rat pretreated with α-LA shows few fibroblastic cells proliferation in the portal area along with some collagen fibers deposition. **G:** Section taken from a liver of rats co-treated with both apocynin and α-LA showing an absence of pseudolobules and minimal collagen fibers deposition. **H:** Liver hydroxyproline content in different groups. Values are given as mean ±S.D. (n = 6). a, b or c: Significantly different from the control, ConA or ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test.

respectively, as compared to the ConA group, which was more significant than that found in either apocynin or α-LA only-pretreated groups (Figure 2). Moreover, ConA-intoxicated group showed a significant increase in NF-κB by 146%, as compared to the control group. Remarkably, co-treatment with apocynin and α-LA revealed a significant decrease in NF-κB by 19% with respect to ConA group (Figure 2).

3.5. *Insulin level*

ConA injection showed a significant increase in serum insulin level by 9.5-fold, when compared to the control rats. Considering co-treatment with both apocynin and α-LA, serum insulin level was reduced as compared to ConA-intoxicated group (by 81%) or the groups treated with either apocynin (by 49%) or α-LA (by 67%) (Figure 2).

3.6. *Liver fibrosis markers*

Masson's trichrome stain showed that collagen fibers were not demarcated around the classical hepatic lobules in liver sections from the control, apocynin or α-LA only-treated groups (Figures 3A, 3B, and 3C). In contrast, the collagen fibers were heavily deposited in sections

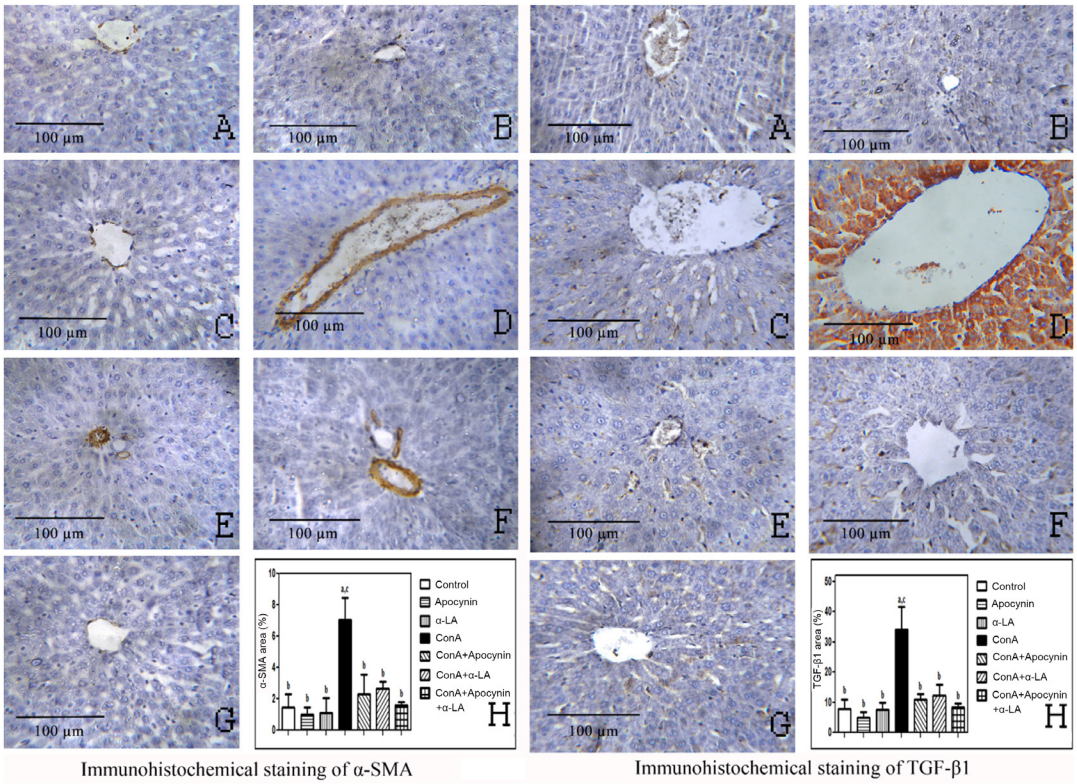

Figure 4. Expression of α-SMA and TGF-β1 antigens by immunohistochemical staining (×400). A, B and C: Sections of livers obtained from rats in control, apocynin or α-LA, respectively, shows that α-SMA and TGF-β1 antigens were minimally detected in the walls of the central vein. **D:** Sections taken from livers of rats intoxicated with ConA shows extensive α-SMA and TGF-β1 expression (brown color). **E:** Sections taken from livers of rats pretreated with apocynin showing mild α-SMA and TGF-β1 expression. **F:** Sections taken from livers of rats pretreated with α-LA shows moderate expression of α-SMA and mild expression of TGF-β1. **G:** Sections taken from livers of rats co-treated with both apocynin and α-LA showing limited α-SMA and TGF-β1 expression (brown color). **H:** Quantitative image analysis expressed as percentage of area of immunopositive reaction. Values are given as mean ±S.D. (n = 10). a, b or c: Significantly different from the control, ConA or ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test. α-SMA, alpha-smooth muscle actin; TGF-β1, transforming growth factor-beta1.

taken from ConA-intoxicated group (Figure 3D), while α-LA pretreated group showed moderate collagen fibers deposition (Figure 3F). Remarkably, pretreatment with either apocynin alone or along with α-LA markedly counteracted these changes (Figures 3E and 3G). As expected, ConA-intoxicated rats showed significantly increased hydroxyproline content by 185%, as compared to the control group. Co-treatment of animals with both apocynin and α-LA revealed reduction in hydroxyproline content by 41%, compared to ConA group (Figure 3H).

Immunohistochemical staining revealed minimal α-SMA and TGF-β1 expression in the liver sections from the control, apocynin or α-LA only-treated rats (Figures 4A, 4B, and 4C). However, ConA-intoxicated group showed significantly raised expression of α-SMA and TGF-β1 by about 4 and 3-fold, respectively, as compared to the control (Figure 4D). Compared with ConA intoxicated group, liver sections of rats pretreated with apocynin alone, α-LA alone or both of them showed a marked reduction in α-SMA expression by about 68, 62 and 77%, respectively, as well as a reduction in TGF-β1 expression by about 68 and 64 and 75%, respectively (Figures 4E, 4F, and 4G). Figure 4H represents the percentage of area of immunopositive reaction.

3.7. *NOX-1 and NOX-4 gene expression*

NOX-1 and NOX-4 mRNA tissue expression showed about 7.5 and 8-fold increase, respectively, in ConA-injected group, as compared to the control. While in the combination group, NOX-1 and NOX-4 mRNA tissue expression were significantly lowered by 59 and 78%, respectively, when compared to the group intoxicated with ConA, which was also more significant than either apocynin or α-LA only-pretreated groups (Figure 5).

4. Discussion

The ConA model is a typical and well established one for investigating T-cell and macrophage dependent liver injury in rodents, which closely resembles the pathogenesis mechanisms of viral and autoimmune hepatitis in humans. ConA is purified from *Canavalia brasiliensis*, after it's *i.v.* injection, hepatic $CD4^+$ T-cells recognize the ConA-modified major histocompatibility complex structures of kupffer cells and become activated, followed by the release of inflammatory mediators in the blood (*18*).

Injection of ConA for six consecutive weeks was

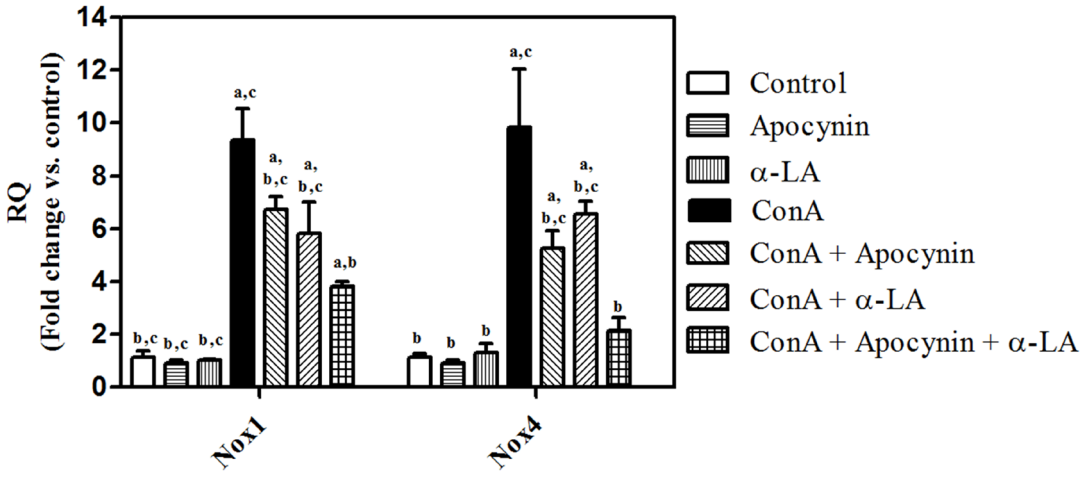

Figure 5. Effect of treatment with 50 mg/kg apocynin and/or 50 mg/kg α-LA on NOX-1 and NOX-4 mRNA expression in ConA-induced liver fibrosis in rats. Values are given as mean ± S.D. (n = 15). a, b or c: Significantly different from the control, ConA or ConA + apocynin + α-LA group, respectively, at $p < 0.05$ using ANOVA followed by Tukey-Kramer as a post-hoc test. NOX-1, NADPH oxidase-1; NOX-4, NADPH oxidase-4.

found to significantly increase serum ALT and AST levels that is attributed to increased enzymes release from damaged liver parenchymal cells into the blood stream (*19*). Meanwhile, serum levels of TC, TG and total bilirubin were significantly increased in ConA-intoxicated group, while albumin level was significantly reduced. Pretreatment of apocynin along with ConA significantly ameliorated these changes. In this context, the hepatoprotective effects of apocynin in a rat model of diet-induced hypercholesterolaemia were reported by a previous study (*8*). Interestingly, the group co-treated with both apocynin and α-LA showed a more pronounced hepatoprotective effect as compared to that treated with apocynin alone. Beside hepatotoxicity indices; histopathological examination revealed severe inflammatory cells infiltration as well as severe fibrosis in the portal area induced by ConA which is in accordance with previous studies (*20,21*). Remarkably, the combination of apocynin and α-LA preserved normal liver tissue architecture with few inflammatory cells infiltration.

Liver fibrosis is characterized by both quantitative and qualitative alteration of hepatic ECM, as a consequence of HSCs activation towards myofibroblast-like cells. This is characterized by increased liver content of α-SMA as a marker for activated HSCs, and accumulation of ECM mainly collagen, which is stimulated by the multifactorial growth factor TGF-β (*22*). In the present study, histopathological examination of collagen fibers using Masson's trichrome stain revealed severe fibroblastic cells proliferation in liver samples of ConA intoxicated group as well as elevated hydroxyproline content. It is known that hydroxyproline is the main characteristic compound in collagen that indicates increased *de novo* synthesis of liver collagen (*23*). Nil fibroblastic cells proliferation was obvious in the liver tissues isolated from rats pretreated with either apocynin alone or together with α-LA. These findings were further confirmed by the decreased content of hydroxyproline within the same groups.

Furthermore, the distribution of α-SMA and TGF-β1 positive hepatic cells were significantly upregulated in the ConA-injected group. Moreover, α-SMA has been directly related to experimental liver fibrogenesis (*24*). Also, the ongoing inflammation in the liver is associated with the formation of the profibrogenic cytokine TGF-β1 (*25*) that has been shown to regulate multiple fundamental cellular processes, including cell growth, migration, adhesion, ECM deposition and apoptosis (*26,27*). The increased α-SMA and TGF-β1 expression was counteracted by pretreatment with either apocynin or α-LA, however, co-treatment with apocynin and α-LA showed a further significant reduction of α-SMA and TGF-β1 expression. Indeed, α-LA reduced them in experimental models of hepatic fibrosis in rodents induced by and dimethylnitrosamine (*28*) and bile duct ligation (*29*).

The next step was to explore the mechanism underlying the hepatoprotective and antifibrotic effects of apocynin and α-LA in combination. More evidence links oxidative stress involvement in ConA-induced liver injury (*30,31*), where SOD, GSH and CAT protects against the deleterious effects of ROS (*32,33*), while MDA is used as an indicator of cellular oxidation status (*34*). In the present study, ConA significantly lowered the liver tissue content of GSH and activities of SOD and CAT while it induced a significant increase in liver MDA content. These results are in accordance with previous studies (*22,35*). Apocynin pretreatment significantly counteracted ConA-induced oxidative stress in accordance with earlier studies performed in the heart and vascular tissue of rats (*36,37*). Paradoxically, pretreatment with α-LA significantly increased only the content of GSH. Meanwhile, co-

treatment with apocynin and α-LA along with ConA proved to be the most effective in significantly counteracting the changes occurring in the assessed oxidative stress markers.

T-cell activation elicited by ConA resulted in the elevation of the cytokines TNF-α and IL-6, which play critical roles in the development of ConA-induced hepatic injury (*38,39*). Furthermore, TNF-α stimulates the release of cytokines and promotes oxidative stress causing liver damage (*40*). Indeed, NF-κB is crucial during hepatic fibrogenesis through regulating hepatocyte injury, inflammatory signals and fibrogenic responses in HSCs (*41*). NF-κB activation in response to liver injury results in production and secretion of proinflammatory cytokines such as TNF-α and IL-6 (*42*). Pretreatment with apocynin succeeded in significantly decreasing the serum levels of TNF-α and IL-6 induced by ConA intoxication. The anti-inflammatory activity of apocynin was supported by previous studies conducted on rodents (*43,44*). Also, α-LA pretreatment with ConA produced a significant reduction of TNF-α and IL-6, which coincides with previous studies on different models of liver injury in rats (*45,46*). Once again concomitant treatment with apocynin and α-LA led to a further significant decrease in the serum levels of TNF-α and IL-6.

In addition, Fartoux *et al*. (*47*) demonstrated that insulin resistance and increased circulating insulin is a cause rather than a consequence of liver fibrosis. Moreover, antioxidants can alleviate insulin resistance through ROS-scavenging activity (*48*). Further, Sukumar, Viswambharan (*49*) identified a NOX enzyme as a contributor in insulin resistance-mediated oxidative stress and postulated that its pharmacological inhibition may possess a novel therapeutic target in insulin resistance-related diseases. In this context, rats injected with ConA showed a significant serum hyperinsulinemia in accordance with Francisco-DoPrado, Zambelli (*50*). Pretreatment with either apocynin or α-LA significantly counteracted the effect of ConA on serum insulin. Earlier studies have reported the effect of apocynin on insulin in a rat model (*48*). Interestingly, co-treatment with both apocynin and α-LA almost restored the normal level of insulin being significantly lower than the groups treated with ConA only or along with apocynin or α-LA.

Finally, we tried to explore the possible role of NOXs in ConA-induced liver hepatitis. It was found that, a significant elevation of liver NOX-1 and NOX-4 gene expression after ConA administration was evident. Thus, NOX enzymes could play a role in ConA induced liver injury. TGF-β in fetal rat hepatocytes was shown to generate ROS through activation of NOX enzymes and down-regulation of antioxidant genes (*51*). This was found to be coincident with the increase in Rac-1 protein level, a well-known activator of NOX-1 (*52*). As a NOX inhibitor, apocynin pretreatment resulted in a significant reduction of NOX-1 and NOX-4 mRNA expression. This protective effect of apocynin coincides with a previous experiment conducted on rat hepatoma cells (*53*). Also, pretreatment with α-LA reduced NOX-1 and NOX-4 expression, which was in agreement with a previous study where α-LA inhibited the activation of NOX and thus, reduced ROS production in *H. pylori*-infected gastric adenocarcinoma AGS cells (*54*). The combining treatment of apocynin and α-LA along with ConA in the present study significantly reduced NOX-1 and NOX-4 expression compared to any of the singly-pretreated groups.

Collectively, this study highlights the crucial role that NOX enzymes play in the pathogenesis of ConA-induced liver fibrosis. It was also shown that using apocynin and α-LA in combination possess a marked antifibrotic effect. This was attributed to the reduction of NOX enzymes in liver tissue, therefore, limiting the production of free radicals, inflammation, insulin and subsequent chronic hepatic fibrosis.

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Original Article

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Effect of *CYP2C9*, *VKORC1*, *CYP4F2*, and *GGCX* gene variants and patient characteristics on acenocoumarol maintenance dose: Proposal for a dosing algorithm for Moroccan patients

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Summary **We investigated the impact of non-genetics factors, and single nucleotide polymorphisms (SNPs) in *VKORC1*, *CYP2C9*, *CYP4F2*, and *GGCX* on acenocoumarol dosage in Moroccan adult's patients, in order to develop an algorithm to predict acenocoumarol dose for Moroccan patients. Our study consisted of 217 Moroccan patients taking a maintenance dose of acenocoumarol for various indications. The patients were genotyped for *VKORC1* –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9*2*, *CYP2C9*3*, *CYP4F2* 1347 G>A and *GGCX* 12970 C>G SNPs. The statistical analysis was performed using the SPSS software. The age and SNPs in *VKORC1* and *CYP2C9* were significantly associated with the weekly acenocoumarol dose requirement ($p = 0.023$, $p = 0.0001$ and $p = 0.001$ respectively). There was no association found between the weekly acenocoumarol dose and the *CYP4F2* or *GGCX* variants (p-value > 0.05). Non-parametric analysis confirmed the accumulate effect of variant alleles at *VKORC1* –1639 G>A, *VKORC1* 1173 C>T and *CYP2C9* SNPs on the acenocoumarol dose requirement. With 90.24% less dose required for one patient carrying homozygote variant at *VKORC1* –1173 (TT) and *CYP2C9* *x/*x haplotype. The multiple linear regression analysis showed that mutation in *VKORC1* –1639, *VKORC1* 1173 SNPs, or in *CYP2C9* haplotype reduces the mean acenocoumarol weekly dose to 25.4%, 23.4% and 6.2%, respectively. The R2 for multiple regression analysis final model was found to be 35.9%. In this work we were able to establish the factors influencing interindividual sensitivity to the anticoagulant therapy that can help physicians to predict optimal dose requirement for long term therapy.**

Keywords: Acenocoumarol, genetics and non genetics factors, algorithm dose, Morocco

1. Introduction

Acenocoumarol and warfarin are oral anticoagulants of the family of vitamin K antagonists. They are the most prescribed in the prevention and treatment of arterial and venous thromboembolic illnesses (*1*). Interindividual variation in response to therapy constitutes a serious problem for determining the appropriate vitamin K antagonists dose (*2*). This variability is known to depend on many environmental and non-genetic factors; nevertheless a genetic factor has also been reported to play an important role in the variability of the appropriate dose and antivitamin K drug metabolism (*3-7*).

Two genes identified as cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase complex subunit 1 (*VKORC1*) are mostly described to influence the pharmacokinetic and pharmacodynamic parameters

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of vitamin K antagonists (*6,8-11*). CYP2C9 is the main enzyme responsible for the biotransformation and subsequent elimination of vitamin K antagonists (*11,12*). Available data indicate that the *CYP2C9* is polymorphic (3 major alleles: *CYP2C9*1* wild-type allele, *CYP2C9*2* and *CYP2C9*3* variant alleles), and its genetic variability has been reported to be related with variations in the levels of enzyme activity (*12-14*). The two defective alleles *CYP2C9*2* and *CYP2C9*3* were characterized as slow metabolizers and known to be strongly associated with dose requirement and bleeding complications of vitamin K antagonist (*12-17*). *VKORC1* code for the target enzyme of coumarins (*18-20*). Vitamin K antagonists exert its effect through inhibition of the *VKORC1* gene product. Genetic variations within *VKORC1* result in changed sensitivity to vitamin K antagonists. Available data indicate that the single nucleotide polymorphism (SNP) in the promoter region at the nucleotide position –1639 G>A for *VKORC1* gene and there intronic polymorphism 1173 C>T were found to be strongly affecting vitamin K antagonists dosage (*21*). These SNPs were found to be in linkage disequilibrium (*22*). Patients carrying the wild type G allele for the *VKORC1* –1639 G>A required a higher dose of vitamin K antagonists compared with those carrying the A variant allele (*23*). Also, patients carrying the wild type C allele for *VKORC1* 1173 C>T required a higher dose of vitamin K antagonists compared with those carrying the variant T allele (*24*).

Recently, the genes of cytochrome P450 4F2 (*CYP4F2*) and gamma glutamyl carboxylase (*GGCX*) also contributed moderately to the variability of vitamin K antagonists dose estimated between 1-2% in Caucasian patients (*25,26*). *CYP4F2* gene encodes an enzyme that metabolizes vitamin K1 to hydroxyvitamin K1 (*25*). Whereas GGCX is the enzyme responsible for gamma carboxylation of vitamin K-dependent proteins (*27*).

Acenocoumarol is mostly prescribed in Europe and Africa. Many works have been undertaken to study the implication of the genetic polymorphism of the *CYP2C9* and *VKORC1* on inter-individual acenocoumarol dose variability. In Morocco, acenocoumarol is the only one vitamin K antagonist drug that has had its marketing authorization. However, only a few small studies have investigated the contribution of the non-genetic factors, *CYP4F2*, *CYP2C9* and *VKORC1* –1639 G>A polymorphisms on acenocoumarol dose requirements (*28,29*). And little information is available on the possible implication of *VKORC1* 1173 or *GGCX* SNPs on the subject in our country. The aim of this study was to evaluate the influence of non-genetic and *CYP2C9*, *VKORC1* –1639, *VKORC1* 1173, *CYP4F2* and *GGCX* polymorphisms on acenocoumarol maintenance dose in a cohort of Moroccan adult patients, in order to develop a useful algorithm for predicting anticoagulant dose for Moroccan patients.

2. Materials and Methods

2.1. *Ethic statement*

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the Faculty of Medicine and Pharmacy of Rabat, Mohamed V University, Morocco following the guidelines set by the Declaration of Helsinki. For each patient, an information sheet has been completed with epidemiological data. Written informed consent was obtained from all patients after being informed of the purpose of our work.

2.2. *Patients and sample collection*

A total of 217 patients from different regions of Morocco were recruited in this study. They were registered between January 2015 to December 2016 in the various clinical departments of the Mohamed V Military Teaching Hospital (MVMTH) at Rabat for the surveillance of Thromboembolic (TE) disease, atrial fibrillation (AF) or heart valves. Patients were 17 years or older and were taking a maintenance dose of acenocoumarol to maintain an International Normalized Ratio (INR) of 2.0-3.0.

Cases were defined as those whose acenocoumarol dose requirement has retained constant for at least four successive clinic visits. All these patients are referred to the laboratory of Hematology in the MVMTH for the realization of the prothrombin time (PT)/INR analysis.
Upon arrival at the Laboratory, a blood sample was collected (4 mL) into a sterile EDTA vacutainer and stored at 4°C until further processing for DNA extraction, and a sample was collected in a sterile citrate vacutainer for INR assessment. Both samples were collected in the same visit; the INR sample was processed on the same day while the DNA samples were referred for genetic testing in the Laboratory of Medical Biotechnology, Faculty of Medicine and Pharmacy of Rabat.

Patients who were on concomitant therapy with drugs potentially interacting with acenocoumarol, patients with abnormal blood tests of renal or hepatic function; lacting women and alcoholics were excluded from the study.

2.3. *DNA extraction*

DNA was extracted from blood samples using the QIAamp DNA Blood Extraction Kit according to the manufacturer's instructions (Qiagen, Germany). DNA was quantified by NanoDrop Analyser (ND 1000) spectrophotometer (NanoDrop Technologies, Wilmington, USA). The ratio of absorbance at 260 and 280 nm of DNA was between 1.7 and 1.9.

2.4. *Amplification and purification*

Amplification reaction of each *VKORC1* –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9*2*, *CYP2C9*3*, *CYP4F2* 1347 G>A and *GGCX* 12970 C>G SNPs was carried

out using the Master Mix 2X (Bioline, London, UK), 400 nM of each appropriate primers (Eurogentec, Belgium) (Table 1), and 60 ng of template DNA in a 25 µL reaction volume under the following conditions: preheating at 95°C for 3 min, then 35 cycles of (95°C, 30 s; 58°C, 30 s; 72°C, 30 s), followed by final extension of 5 min at 72°C employing a thermal cycler. The PCR amplicons of *VKORC1*, *CYP2C9*, and *CYP4F2* gene were electrophoresed on a 3% agarose gel and on a 1% agarose gel for *GGCX* gene.

2.5. *Genotyping*

After the PCR reaction, the amplification products were purified using ISOLATE II PCR and Gel Kit according to the manufacturer's instructions (Bioline, London, UK). The purified amplicons were genotyped for all investigated SNPs using Restriction Fragment length Plymorphism (RFLP) technique. So, the purified amplicons was digested with 2 units of restriction enzyme (Biolabs, New England) overnight at 37°C. The digested products were analyzed on 3% agarose gel except for *GGCX* 12970 C>G polymorphism who were separated on 2% agarose gel. The restriction enzymes used for each SNP and the size of digested products are summarized in Table 1.

2.6. *Statistical analysis*

Statistical analysis was performed using SPSS software (Version 18) under windows. Hardy Weinberg equilibrium was determined using a web-based calculator available at (*https://www.snpstats.net/snpstats/start.htm*) (*30*). The effect of non-genetic and genetic variants on the mean acenocoumarol dose was calculated by univariate analysis using appropriate test depending on the nature of the variables compared. Kruskal-Wallis or Mann-Whitney test was used for non-parametric analysis where appropriate. For all statistical tests, the significance level was set at 0.05. A multiple linear regression analysis was used to assess the ability of age, *VKORC1* (1173) and (1639) genotypes and *CYP2C9* haplotype to predict the dose of acenocoumarol in order to propose to clinicians an algorithm taking into account all these variables.

3. Results

3.1. *Patients characteristics*

Two hundred and seventeen patients, receiving stable dose of acenocoumarol treatment, were included in this study. The average age of the study populations was 57 ± 14 years (17-87 years old), the sex-ratio was 0.95 (111 females and 106 males), and the mean of acenocoumarol maintenance dose was 22.94 ± 12.01 mg/week. The characteristics of the included patients are summarized in the Table 2. Genotypic frequencies of all genes assessed in this study are given in Table 3. Allelic frequencies for the all assessed SNPs were found to be in Hardy-Weinberg equilibrium ($p > 0.05$) (data not shown).

Table 1. Primer design, restriction enzyme used and DNA fragments found for *VKORC1*, *CYP2C9*, *CYP4F2* and *GGCX* variations

Genetic polymorphism	Primer sequences	PCR product size	Restriction enzyme (RE)	RE digestion product size
VKORC1 (1639 G>A)	F5'GAGCCAGCAGGAGAGGGAAATAT 3' R-5'GTTTGGACTACAGGTGCCTGCC 3'	291 bp	Msp I	WT-167 + 124 bp Htz-291 + 167 + 124 bp Mut-291 bp
VKORC1 (1173 C>T)	F-5'CTAAGATGAAAAGCAGGGCCTAC3' R-5'CTGCCCGAGAAAGGTGATTTCC3'	201 bp	Sty I	WT-127 + 74 bp Htz-201 + 127 + 74 bp Mut-201 bp
*CYP2C9*2 (430 C>T)*	F-5'TCCTAGTTTCGTTTCTCTTCCTGT3' R-5'ATAGTAGTCCAGTAAGGTCAGTGA3	221 bp	Ava II	WT-122 + 99 bp Htz-221 + 122 + 99 bp Mut-221 bp
*CYP2C9*3 (1075 A>C)*	F-5'CACGAGGTCCAGAGATGCATTG3' R-5'CTTCGAAAACATGGAGTTGCAGT3'	135 bp	Nsi I	WT-116 + 19 bp Htz-135 + 116 + 19 bp Mut-135 bp
CYP4F2 (1347 G>A)	F-5′TGAAGGAGGCCTTCTCCTGACTG3′ R-5′CCAGCCTTGGAGAGACAGACAG3′	232 pb	PvuII	WT-146pb + 86 bp Htz-232 + 146pb + 86 bp Mut- 232 bp
GGCX (12970 C>G)	F-5'GCTTCTTGTTGCGAAAGCTCTAT3' R-5'CAAACACTTGGGAACAGTTAGCT3'	1288bp	Hind III	WT-1206+82 Htz-1288+ 1206pb + 82 bp Mut- 1288 bp

WT, wild type; Htz, heterozygous; Mut, homozygous mutant; bp, base pairs.

3.2. *Association of non-genetic factors with acenocoumarol dose*

The associations between age, sex, body mass index (BMI), ethnic origin, acenocoumarol therapy indication, duration of therapy, medications, the good diet monitoring and Acenocoumarol weekly dose were studied (Table 2). Univariate analysis showed that the age was the only one non-genetic factor significantly associated with the acenocoumarol weekly maintenance dose (p = 0.023) (Table 2). Patients up to 65 years (17.41 ± 10.80 mg/week) of age required a lower dose compared to the patients under 65 years old (25.25 ± 11.76 mg/week) (data not showed).

Table 2. Baseline characteristics of study population of the patients (*n* = 217) receiving Acenocoumarol therapy and the relationship of these characteristics to the maintenance weekly dose in univariate analysis

Patient details	*n* (%) or mean ± SD [min-max]*	*P* value
Age (Years)	57 ± 14 [17-87]	
< 65 Years	153 (70.5)	0.023
> 65 Years	64 (29.5)	0.000
Sex		0.056
Male	106 (48.84)	
Female	111 (51.15)	
Weight (kg)	71.85 ± 11.85	0.747
Height (cm)	169.12 ± 17.80	0.871
Body mass index (Kg/m^2)	24.52 ± 4.15 [13,95-46,77]	0.531
Ethnic		0.973
Arabic	147 (67.7)	
Berber	64 (29.5)	
Sahara	6 (2.8)	
Indication for Acenocoumarol therapy		0.321
TE disease	62 (28.58)	
AF	30 (13.82)	
Heart valves	125 (57.60)	
Duration of the therapy (Years)	6.99 ± 6.07 [1 –35]	0.56
Medication	130 (59.90)	0.131
Good diet monitoring	175 (80.64)	0.212

*Values given are mean ± standard deviation or number of participants (*n*) (%).

Table 3. Genotypic and allelic distribution of *CYP2C9*, *VKORC1*, *CYP4F2* and *GGCX* SNPs and relationship with weekly dose (mg) of Acenocoumarol

Gene and polymorphism	*n* (%)	Allele frequency	Mean dose (mg/Week) ± SD, Difference (95% CI)		*p*-value*
VKORC1-1639 G>A					
GG	57 (26.26)	G 0.54	32.00 ± 14.42	Ref.	0.0001
GA	120 (55.29)	A 0.46	21.40 ± 8.61	-10.60 (-13.90 – -7.30)	
AA	40 (18.43)		14.65 ± 8.75	-17.35 (-21.58 – -13.12)	
VKORC1-1173 C>T					
CC	64 (29)	C 0.55	32.66 ± 13.52	Ref	0.0001
CT	111 (51)	T 0.45	19.66 ± 7.93	-13.00 (-16.14 – -9.85)	
TT	42 (19)		16.81 ± 9.74	-15.85 (-19.82 – -11.87)	
*CYP2C9 *2*					
CC	161 (74.19)	C 0.87	24.56 ± 12.52	Ref	0.002
CT	55 (25.34)	T 0.13	18.49 ± 8.96	-6.07 (-9.66 – -2.48)	
TT	1 (0.01)		7	-17.56 (-40.61 – 5.49)	
*CYP2C9 *3*					
AA	191 (88.01)	A 0.94	23.48 ± 12.09		0.075
AC	26 (11.99)	C 0.06	19.00 ± 10.89	-4.48 (-9.37 – 0.42)	
*CYP2C9 Haplotypes***					
*1/*1	144 (66.35)	*1 0.81	25.05 ± 12.51	Ref	0.001
*1/*x	65 (29.95)	*x 0.19	19.05 ± 9.96	-6.00 (-9.43 – -2.58)	
*x/*x	8 (0.036)		16.63 ± 8.42	-8.42 (-16.74 – -0.10)	
CYP4F2					
GG	82 (37.78)	G 0.63	22.72 ± 12.58	Ref	0.701
GA	108 (49.76)	A 0.37	23.19 ± 11.25	0.47 (-3.00 – 3.93)	
AA	27 (12.44)		22.63 ± 13.60	-0.09 (-5.34 – 5.16)	
GGCX					
CC	205 (94.47)	C 0.97	23.05 ± 12.02	Ref	0.379
CG	12 (5.53)	G 0.03	21.00 ± 12.34	-2.05 (-9.06 – 4.95)	

Values given are number of patients (*n*) (%). *Bold indicates statistical significance. ***CYP2C9* haplotypes: *1/*1, wild-type homozygotes; *1/*x: *CYP2C9*2* or *CYP2C9*3* heterozygotes (*1/*2 and *1/*3); and *x/*x: *CYP2C9*2* homozygotes (*2/*2) or multiple heterozygotes (*2/*3).

3.3. *Association of VKROC1, CYP2C9, CYP4F2 and GGCX SNPs with the dose of acenocoumarol*

Two SNPs were investigated in the *VKORC1* gene (Table 1). Both *VKORC1* –1639 and *VKORC1* 1173 polymorphisms were observed to be affecting the weekly acenocoumarol dose significantly (p = 0.0001) (Table 3). Carriers of the GG genotype from *VKORC1* –1639 SNP requiring the highest dose (32 ± 14.42 mg/week) compared to GA carriers (21.4 ± 8.61 mg/week) and AA carriers (14.65 ± 8.75 mg/week) (Table 3). Also carriers of the CC genotype of *VKORC1* 1173 SNP requiring the highest dose (32.66 ± 13.52 mg/week) compared to CT carriers (19.66 ± 7.63 mg/week) and TT carriers (16.81 ± 9.74 mg/day) (Table 3).

Five variants (allelic combinations at two loci) were detected in Moroccan participants included in this work. A significant association was observed between the weekly maintenance acenocoumarol dose and the *CYP2C9* variants (p = 0.001) (Table 3). Patients carrying wild-type *CYP2C9* allele were found to require higher dose (25.05 ± 12.51 mg/week) than those with *CYP2C9* variant allele (19.05 ± 9.96 mg/week for the *CYP2C9* heterozygotes (*1/*2, *1/*3, *2/*3) and 16.63 ± 8.42 mg/week in mutant homozygotes patients (*2/*2) (Table 3).

There was no association found between the weekly acenocoumarol dose and the *CYP4F2* or *GGCX* variants (p-value > 0.05) (Table 3).

3.4. *Cumulate effect of VKORC1 and CYP2C9 polymorphisms on acenocoumarol dose*

The relationship between the three polymorphisms of each two SNPs of *VKORC1* gene (–1639 and 1173) and *VKORC1* –1639 or *VKORC1* 1173 variants with

Table 4. Effect of genotype combination (VKORC1 1639 and VKORC1 1173) on mean weekly dose of acenocoumarol

VKORC1 1639 G>A	*VKORC1 1173 C>T*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
GG	CC	50	34.12 ± 11.49		0.001
	CT	7	16.86 ± 9.28	-17.26 (-25.14 – -9.38)	
GA	CC	13	26.85 ± 3.11	-7.27 (-13.35 – -1.20)	0.111
	CT	91	20.55 ± 6.71	-13.57 (-17.01 – -10.13)	
	TT	16	21.81 ± 7.64	-12.31 (-17.92 – -6.70)	
AA	CC	1	35	-0.88 (-18.84 – 20.60)	0.282
	CT	13	14.92 ± 8.74	-19.20 (-25.28 – -13.12)	
	TT	26	13.73 ± 5.24	-20.39 (-25.11 – -15.67)	

Table 5. Effect of genotype combination (*VKORC1* 1639 and *CYP2C9* haplotypes) on mean weekly dose of acenocoumarol

VKORC1 1639 G>A	*CYP2C9 Haplotype*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
GG	*1/*1	42	34.76 ± 14.63		0.047
	*1/*X	13	23.69 ± 11.61	-11.07 (-17.40 – -4.73)	
	*X/*X	2	28	-6.76 (-21.21– 7.68)	
GA	*1/*1	76	22.66 ± 6.94		0.029
	*1/*X	40	19.60 ± 6.53	-3.06 (-6.96 – 0.84)	
	*X/*X	4	15.50 ± 6.50	-7.16 (-17.40 – 3.08)	
AA	*1/*1	26	16.35 ± 6.14	-18.42 (-23.40 – 13.44)	0.027
	*1/*X	12	12.17 ± 5.91	-4.18 (-11.15 – 2.79))	
	*X/*X	2	7.5 ±3 .50	-8.85 (-23.49 – 5.80)	

Table 6. Effect of genotype combination (*VKORC1* 1173 and *CYP2C9* haplotypes) on mean weekly dose of acenocoumarol

VKORC1 1173 G>A	*CYP2C9 Haplotype*	*n* = 217 (%)	Mean dose (mg/Week) ± SD	Difference (95% CI)	*p*-value
CC	*1/*1	48	41 ± 12.81		0.011
	*1/*X	13	23 ± 7.77	-11.19 (-17.24 – -5.14)	
	*X/*X	3	11	-12.17 (-23.68 – -0.65)	
CT	*1/*1	76	24 ± 7.88		0.155
	*1/*X	40	21 ± 7.83	-1.38 (-5.25 – 2.48)	
	*X/*X	7	20 ± 5.19	-5.61 (-15.57 – 4.35)	
TT	*1/*1	29	18.59 ± 6.14		0.028
	*1/*X	12	13.58 ± 5.91	-5.00 (-11.64 – 1.64)	
	*X/*X	1	4	-14.59 (-34.27 – 5.09)	

CYP2C9 haplotypes and weekly acenocoumarol dose were analyzed, significant difference observed between the different combinations using non-parametric analysis was given in Table 4, 5 and 6. Carriers of the *VKORC1* –1639 GG and *VKORC1* 1173 CC required the highest weekly dose among all genotype combinations (42 ± 11.49 mg/week), whereas carriers *VKORC1* 1173 TT and *CYP2C9* mutant homozygotes or compound heterozygotes (*CYP2C9* *x/*x) required the lowest daily dose (4 mg/week).

3.5. *Multiple linear regression analysis*

The mean weekly acenocoumarol dose was transformed into a logarithmic dose and used as a dependent variable in univariate and multivariate analysis with age, *VKORC1* –1639, *VKORC1* 1173 genotypes and *CYP2C9* haplotype.

The results of regression analyses are summarized in Table 7. Mutation in *VKORC1* –1639, *VKORC1* 1173 SNPs, or in *CYP2C9* haplotype reduces the weekly mean dose of acenocoumarol at 25.4%, 23.4%, and 6.2% respectively. Considering all these genetic factors the R^2 for multiple regression analysis model was found to be 33.7%. Whereas the R^2 for multiple regression analysis final model was 35.9% when age variant had combined to the regression analysis with genetic factors (Table 7).

From the data in Table 7, the proposed regression equation to predict weekly dose (WD) of acenocoumarol for our final Model was:

Log (WD) = 1.925 – 0.108x (*VKORC1* 1639 G>A) – 0.073x (*VKORC1* 1173 C>T) –0.093x (*CYP2C9* Haplotype) – 0.003x (Age)

4. Discussion

The aim of this study was to investigate the impact of non-genetics factors and *VKORC1*, *CYP2C9*, *CYP4F2*, and *GGCX* polymorphisms on acenocoumarol maintenance dose in a cohort of Moroccan patients. Acenocoumarol is the only one coumarin anticoagulants available for the treatment and prevention of thromboembolic diseases in Morocco. Several factors have been described to influence the individual sensitivity for anticoagulant therapy. Genetic factors, mostly SNP variations in *CYP2C9* and *VKORC1*, in association with non-genetic factors have been reported to account for 60% of the variation in vitamin K antagonists dosage (*6*).

In our analysis, the age was the only one non-genetic factor significantly associated with the acenocoumarol dose and the required dose was significantly lower when age was increased. However, many studies have reported that the acenocoumarol and warfarin dose was influenced by other demographic factors such as gender, body mass index and ethnicity (*6,23*).

Our study showed a significant association between genetic polymorphisms in *VKORC1* and *CYP2C9* and acenocoumarol dose requirement, and corroborating with the findings reported in other countries including Morocco (*6-14,17,21,23,28,29*).

Several studies published in the world show that *VKORC*1 –1639 G>A, *VKORC1* 1173 C>T, *CYP2C9**2

Table 7. Multiple regression models considering log-transformation the weekly dose of AC as dependent variable

Model	Composition	Unstandardized coefficients		Standardized coefficients	*p* value	95% Confidence Interval for B		Model summar		
		B	SE	Beta		Lower Bound	Upper Bound	R	R^2	Adjusted R^2
1	Constant	1.641	0.042		-	1.558	1.723			
	VKORC1 -1639	-0.176	0.021	-0.504	0.000	-0.217	-0.136	0.504	0.254	0.250
2	Constant	1.610	0.041		-	1.530	1.690			
	VKORC1 1173	-0.163	0.020	-0.483	0.000	-0.202	-0.123	0.483	0.234	0.230
3	Constant	1.464	0.046		-	1.374	1.554			
	CYP2C9 Haplotype	-0.128	0.034	-0.249	0.000	-0.196	-0.061	0.249	0.062	0.057
4	Constant	1.802	0.051		-	1.701	1.902			
	VKORC1 -1639	-0.106	0.029	-0.303	0.000	-0.163	-0.049	0.580	0.337	0.327
	VKORC1 1173	-0.085	0.028	-0.252	0.003	-0.139	-0.030			
	CYP2C9 Haplotype	-0.099	0.023	-0.235	0.000	-0.145	-0.052	0.599	0.359	0 .347
Final	Constant	1.925	0.068		-	1.791	2.059			
model	*VKORC1 1639*	-0.108	0.028	-0.309	0.000	-0.165	-0.052			
	VKORC1 1173	-0.073	0.028	-0.218	0.090	-0.128	-0.019			
	CYP2C9	-0.093	0.023	-0.221	0.000	-0.138	-0.047			
	Age	-0.003	0.001	-0.152	0.007	-0.004	-0.255			

Stepwise multivariate linear regression algorithm for prediction of stable acenocoumarol weekly dose:
Model 1: Log (WD) =1.641 – 0.176x (*VKORC1* 1639 G>A)
Model 2: Log (WD) =1.610 – 0.163x (*VKORC1* 1173 C>T)
Model 3: Log (WD) =1.464 – 0.128x (*CYP2C9* haplotype)
Model 4: Log (WD) =1.802 – 0.106x (*VKORC1* 1639 G>A) – 0.085x (*VKORC1* 1173 C>T) – 0.099x (*CYP2C9* haplotype)
Final Model: Log (WD) =1.925 – 0.108x (*VKORC1* 1639 G>A) – 0.073x (*VKORC1* 1173 C>T) – 0.093x (*CYP2C9* haplotype) – 0.003x (Age)
- Value 1 for GG; 2 for GA and 3 for AA of *VKORC1* (–1639 G>A)
- Value 1 for CC; 2 for CT and 3 for TT of *VKORC1* (1173C>T)
- Value 1 for *1/*1; 2 for *1/*2 or *1/*3 and 3 for *2/*2 or *2/*3 of *CYP2C9* haplotype
- Age in years

and *CYP2C9*3* were the most significant SNPs to affect vitamin K antagonists dosage (*6-14,17,21,23,28,29*). Our results corroborate with these facts; patients presented homozygous form of wild type allele for *VKORC1* –1639 G>A (genotype GG), *VKORC1* 1173 C>T (genotype CC), or *CYP2C9* haplotype (genotype *1/*1) required a higher acenocoumarol dose than patients in the heterozygous or homozygous variant genotypes groups. Patients with one *VKORC1* –1639 A allele need a 33.12% less dose and patients with two *VKORC*1 –1639 A alleles need a 54.21% less dose compared to patients without allele variant. This effect was also found in other populations (*23*). Similar percentages were found in Caucasian patients (25% and 50% less doses, respectively), while in Asian patients the percentages were lower (14% and 38%, respectively).

According to the previous studies investigated the effect of *VKORC1* –1639 polymorphisms on *VKORC1* expression, homozygotes for the G allele have a higher transcription activity of 44 % than those homozygote for the A allele (*31*). Therefore, homozygotes for the A allele would have lower levels of *VKORC1* expression and logically would require a lower concentration of acenocoumarol, as is the case in our study.

To our knowledge, this is the first study that reports the effect of genetic polymorphisms of the *VKORC1* 1173 C>T SNP in the variability to the acenocoumarol response among Moroccan population.

VKORC1 1173 CC genotype was associated with a significantly higher acenocoumarol dose when compared to both the CT heterozygous genotype and the TT variant genotype. Patients carrying CT or TT genotypes required a 39.80% and 48.53% less dose, respectively, compared to patients with a wild genotype (CC genotype). These results are demonstrated in other countries using acenocoumarol, in the German and Austrian populations (25% and 52%) (*32*), in Dutch patients (28% and 47%) (*24*), and in Chinese patients (33% and 55%) (*33*).

Like Warfarin, acenocoumarol is metabolized by *CYP2C9*. It has been identified that *CYP2C9*2* and *CYP2C9*3* variants alleles reduce in vitro enzymatic activity. Therefore, patients carrying heterozygous and homozygous variants *CYP2C9* alleles were more sensitive to vitamin K antagonists therapy and present a higher risk of bleeding complications than patients carrying the *CYP2C9*1* alleles (*34*). Here, patients with one *CYP2C9* variant allele (*1/*2 or *1/*3) required a 23.95% less dose and patients with two variant alleles (*2/*2, *2/*3) a 33.61% less dose than patients without this variant allele (*1/*1). These results correlate with previous findings indicating that acenocoumarol dose requirement is 19-29% less in carriers one or two *CYP2C9* variant allele than in wild-types (*35*).

The data reported by Smires *et al*. has shown that, there was no association between the acenocoumarol dose variation and the *CYP4F2* polymorphisms among Moroccan population (*28*). Although, the *CYP4F2* variant allele (A) frequency was of 37% (Table 3), our findings support this fact and confirm that the required acenocoumarol dose, is probably not affected by the modification of the *CYP4F2* gene in the Moroccan population.

This study is the first in Morocco to assess whether the genetic polymorphisms of *GGCX* SNP influences the individual response for AC therapy. However, our results shown that there was no association between *GGCX* polymorphisms and acenocoumarol dose maintenance.

The *GGCX* wild type allele (C) was found in a very higher frequency (97%) in our study samples (Table 3). It's known that ethnic differences in allelic frequencies of SNPs implicated in vitamin K antagonists dose variability affect the sensitivity to treatment among different population (*36*). Thus, we can't confirm that the lower frequency of *GGCX* variant allele (G) was responsible for such a result.

However, a large and representative investigation is necessary to better evaluate the effect of *CYP4F2* and *GGCX* SNPs on the acenocoumarol dose assessment among Moroccan population.

Similar to the results from other studies (*12,37*), non-parametric analysis based on acenocoumarol dose, *VKORC1* –1639, *VKORC1* 1173 SNPs and *CYP2C9* haplotypes demonstrated that these three SNPs exert a cumulative effect on the acenocoumarol dose requirement. Patients carrying homozygous variant alleles at *VKORC1* –1639 (AA) and *VKORC1* 1173 (TT) SNPs required 59.75% less dose than wild type for the two SNPs. Carriers *VKORC1* –1639 AA and *CYP2C9* *x/*x genotypes required 78.42% less dose than *VKORC1* –1639 GG and *CYP2C9 *1/*1* genotypes. The single patient who had *VKORC1* –1173 TT variant and *CYP2C9* *x/*x haplotype (*CYP2C9* *2/*2 or *2/*3) had the lowest weekly AC dose (4 mg/week) among the all investigated patients (90.24% less dose).

As reported in other investigations (*33,38-40*), the multiple linear regression analysis confirmed that *VKORC1* and *CYP2C9* contribute mainly to the interindividual variability of the acenocoumarol dose requirement. Our multivariate model including *VKORC1* –1639, *VKORC1* 1173 genotypes and *CYP2C9* haplotype accounted for 33.7% (R^2 = 0.337) of our total observed variation. While our multivariate final model including age, *VKORC1* –1639, *VKORC1* 1173 and *CYP2C9* was 35.9%. The same R^2 value is approximately reported by Dhakchinamoorthi *et al*. (30.4%) (*41*) and Rathore *et al*. (41%) (*42*). However, high values were explained in the algorithms developed by Borobia *et al*. (61%) (*39*) and Markatos *et al*. (55%) (*43*) (Table 8). We can speculate that the R^2 of the multivariate regression model is affected by the nature and number of independent variables.

As shown by the results of this and other studies (*6-14,17,21,23,28,29*), the variation in acenocoumarol dose response is partially influenced by the differences

Table 8. Published algorithms to predict the required acenocoumarol dose

Reference	Country	Genetic parameter	Clinical parameter	R^2 %
Cerezo-Manchado J *et al*. 2013 (*40*)	Spain	*CYP2C9, VKORC1, CYP4F2*	Age, BSA, gender	50
Dhakchinamoorthi K *et al*. 2013 (*41*)	India	*CYP2C9 VKORC1*	Age BMI	30.4
Rathore SS *et al*. 2012 (*42*)	India	*CYP2C9, VKORC1, CYP4F2, GGCX*	Age, weight, height, BSA, gender	41
Borobia A *et al*. 2012 (*39*)	Spain	*CYP2C9, VKORC1, CYP4F2, APOE*	Age, BMI, CM	61
Markatos C *et al*. 2008 (*43*)	Greece	*CYP2C9 VKORC1*	Age, gender, CM	55
Van Schie R *et al*. 2011 (*38*)	Netherlands	*CYP2C9 VKORC1*	Age, height, weight, gender, CM	53
Present Study 2017	Morocco	*CYP2C9 VKORC1*	Age	35.9

in genetic polymorphisms, especially in *CYP2C9* and *VKORC1*. Therefore, our proposed regression equation might be useful to Moroccan clinician physicians for initial determination of acenocoumarol dose. The effectiveness of this equation should be evaluated in a future study.

In conclusion, the present study confirmed that the high variability in the maintenance dose of acenocoumarol in Moroccan populations is related to demographic factors and genetic factors, in particular *VKORC1* –1639, *VKORC1* 1173, *CYP2C9*2* and *CYP2C9*3*. In addition, acenocoumarol susceptibility was shown to be higher in patients with one or more mutations in these SNP. The presence of these mutations indicates that a lower initial dose of acenocoumarol should be used to reduce the risk of bleeding. Furthermore, this study provides a useful model for predicting the weekly dose of acenocoumarol for Moroccan patients based on their genetic makeup.

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Original Article

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Cancer incidence and mortality patterns in Luwan district of Shanghai during 2002-2011

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Summary **Cancer has become the leading cause of death and a major burden to public health in China. The current study analyzed the composition, incidence, mortality, and temporal trends for some major cancer types among permanent residents in Luwan district of Shanghai from 2002 to 2011, so as to provide data for cancer research. Data were collected from the database of cancer registration and management system in Shanghai. Number of new cases, number of deaths, incidence, and mortality of each cancer type were calculated. The incidence and mortality rates were standardized. Temporal trends in the incidence and mortality were assessed using average annual percent change. There were 12,843 new cancer cases and 8,331 deaths from cancer in Luwan from January 2002 to December 2011. Age-standardized incidence rates by Segi's standard were 229.46 and 205.05 per 100,000 population for males and females, respectively. For males, the most commonly diagnosed cancers were lung, colorectal, stomach, liver, prostate, bladder, pancreas, kidney, lymphoma, and esophageal cancers; for females, they were breast, colorectal, lung, stomach, thyroid, liver, ovary, pancreas, uterus, and brain cancers. The incidence rates for all cancers combined increased significantly for both males and females from 2002 to 2011 ($p < 0.05$ for both). Age-standardized mortality rates were 147.04 and 90.62 per 100,000 population for males and females, respectively. The mortality rates have stayed stable during the 10-year period for both males and females ($p > 0.05$ for both). Our results suggest that cancer incidence and mortality rates in Luwan district of Shanghai vary by age, sex, tumor type. The increasing trends in cancer incidence call for effective prevention and control measures in the district. The significance of cancer registration for disease surveillance and management needs to be further advocated.**

Keywords: Cancer, incidence, mortality, temporal trend, Shanghai

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1. Introduction

Cancer is a major public health problem in China and has become the leading cause of death since 2010 (*1*). This increase has been attributed in part to lifestyle changes associated with rapid economic development. Shanghai is the forerunner of China's urbanization and socioeconomic development. There have been tremendous changes occurring in Shanghai in recent decades, which lead to environmental changes such as air and water pollution, and lifestyle changes including

westernized diet, physical inactivity, and reproductive changes (such as late marriage and late childbearing). Luwan district, located at the southeastern part of urban Shanghai, has a total area of 8.05 km^2 and a population around 0.3 million (*2*). The relatively stable and dense aging population, optimized and upgraded industrial structure, and relatively abundant medical resources in this district assume greater representative of developed urban regions in China. Using integrated data based on the district cancer registry in an urban setting, this study provided comprehensive regional information of the cancer burden in the past 10 years.

In the current study, we comprehensively analyzed composition, incidence and mortality of cancer in Luwan district of Shanghai. All cancers and cause-specific incidence and mortality rates were calculated, and further stratified by gender, age categories, and leading causes of cancer occurrence and death. The trends of incidence and mortality rates for all cancers combined and selected cancers were tested during the period of 2002 to 2011. The purposes of this study include: 1) present the regional burden and distribution of cancer in Luwan district of Shanghai, 2) provide population-based evidence for cancer research, future policy design, and health resource allocation, and 3) demonstrate the importance of cancer registration for disease surveillance and management.

2. Materials and Methods

2.1. *Data sources*

The cancer data from January 1st 2002 to December 31st 2011 of permanent residents in Luwan district were collected from the database of cancer registration and management system in Shanghai. The population data were from Center for Disease Control and Prevention of Luwan district in Shanghai. All cancer cases were classified according to the International Classification of Diseases, 10th revision (ICD-10). Because of data corrections or case capture lags, cancer incidence rates were adjusted for reporting delays whenever possible. Percentages of cancers morphology verified (MV%) and death certificate only (DCO%) showed overall data quality is good.

2.2. *Statistical analyses*

Incidence and mortality rates were calculated as the total number of new cases/deaths each year divided by the corresponding annual average population in Luwan district and expressed per 100,000 population. The rates were standardized by the demographic composition developed in the Fifth Nationwide Census in the year 2000, and the Seig's world standard (*3,4*). Age-specific incidence and mortality were also calculated for all cancers combined and selected common cancer types. Gender difference was compared using the u-statistics for two observed Poisson variables. All above analyses were two-sided and performed using EXCEL2007 and SPSS 16.0 (SPSS, Inc., Chicago, IL). A $p < 0.05$ was considered statistical significant.

Temporal trends of the incidence and mortality rates for all cancers combined and the 10 most common cancer types stratified by sex were assessed using fitting joinpoint model by Join-point Regression Program 3.5.1 (*5*). Rates (per 100,000 population) were age-standardized according to the Segi's world standard population and log-transformed. Models were restricted to a maximum of 2 joinpoints (*i.e.*, 3 line segments), and trends were expressed as an annual percent change (APC). The statistical significance of the APC was assessed by the Z test. Terms "increase" or "decrease" were used to describe statistically significant ($p < 0.05$) APC, while the term "stable" was used for non-statistically significant trends.

3. Results

3.1. *Cancer incidence*

The annual number of new cases and incidence for all cancers are presented in Table 1. A total of 12,843 new cancer cases were diagnosed from January 2002 to December 2011. There were 6,563 (51.10%) male cancer cases, and 6,280 (48.90%) female cancer cases. The male to female ratio was 1.05:1. For all cancers combined, the crude incidences of males and females were 417.62 and 391.63 per 100,000 population, respectively. Age-standardized rates by the 2000 Chinese standard population were 191.10 and 180.11 per 100,000 population for males and females, and age-standardized rates by Segi's standard were 229.46 and 205.05 per 100,000 population for males and females, respectively. Male incidence for all cancers combined was higher than the female incidence, and the difference was statistically significant ($u = 3.65$, $p < 0.01$).

The number of new cases, crude and age-standardized incidences for commonly diagnosed cancer types by sites are presented in Table 2. The 10 most common cancer types among males were: lung, colorectal, stomach, liver, prostate, bladder, pancreas, kidney, lymphoma, and esophageal cancers, accounting for about 80 percent of all new cancer cases. Lung cancer alone was accounting for about 19 percent of all new cancer cases in males. The corresponding cancers among females were: breast, colorectal, lung, stomach, thyroid, liver, ovary, pancreas, uterus, and brain, accounting for more than 76 percent of all cases. Breast cancer alone was accounting for approximately 19 percent of all new cancer cases in females (Table 2). The incidences of lung ($u = 13.46$, $p < 0.01$), stomach ($u = 9.88$, $p < 0.01$), liver ($u = 11.96$, $p < 0.01$), bladder ($u = 9.60$, $p < 0.01$), kidney ($u = 5.38$, $p < 0.01$), esophageal ($u = 8.05$, $p < 0.01$) cancers,

Table 1. New cases and incidence of all malignant tumors in Luwan district of Shanghai, 2002-2011

Year	Males					Females					Total				
	New cases	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)	New cases	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)	New cases	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)
2002	654	169034	386.90	188.70	227.65	582	171089	340.17	158.87	184.97	1236	340123	363.40	171.12	202.97
2003	605	164522	367.73	180.63	216.73	585	166582	351.18	164.63	185.79	1190	331104	359.40	170.39	198.47
2004	658	162074	405.99	187.74	228.07	572	164138	348.49	163.56	184.08	1230	326212	377.06	172.66	201.97
2005	586	159090	368.34	175.75	213.54	607	161257	376.42	174.51	202.79	1193	320347	372.41	173.76	206.58
2006	666	156367	425.92	195.50	234.11	592	158819	372.75	178.37	200.40	1258	315186	399.13	184.4	214.03
2007	631	154860	407.46	189.63	224.09	615	157770	389.81	170.83	193.60	1246	312630	398.55	178.69	206.92
2008	641	153593	417.34	185.99	221.81	635	157235	403.85	188.11	217.88	1276	310828	410.52	185.09	217.40
2009	681	152216	447.39	193.40	228.66	601	156526	383.96	175.73	199.44	1282	308742	415.23	183.04	212.11
2010	719	150506	477.72	194.86	238.15	738	155387	474.94	208.56	233.54	1457	305893	476.31	200.58	234.26
2011	722	149281	483.65	211.36	252.71	753	154752	486.58	217.76	244.80	1475	304033	485.14	214.01	248.13
Total	6563	1571543	417.62	191.10	229.46	6280	1603555	391.63	180.11	205.05	12843	3175098	404.49	183.68	214.83

*Age-standardized rate by Chinese standard population based on the result of national census in 2000. †Age-standardized rate by Segi's world standard population.

lymphoma ($u = 8.05$, $p < 0.01$) and leukemia ($u = 2.37$, $p < 0.01$) were significantly higher among males than females. While the incidences of thyroid ($u = 9.46$, $p < 0.01$), brain ($u = 1.84$, $p < 0.05$), and gallbladder ($u = 4.77$, $p < 0.01$) cancers were significantly higher among females than males. There were no significant differences for colorectal ($u = 0.62$, $p > 0.05$), and pancreas ($u = 1.27$, $p > 0.05$) cancers between males and females.

The age distributions of the top 5 most frequently diagnosed cancer types by sex are presented in Figure 1a and 1b. In general, incidences of the 5 most common cancer types among males increased with age (Figure 1a). For lung, colorectal, stomach, and liver cancers, the incidences gradually increased after 45 years of age, and peaked at age group 80-85. There was a sharp increase for lung, colorectal, and stomach cancers after age 55. For prostate cancer, incidence increased steeply after age 70, and peaked at age group 85+. Among females, thyroid cancer was diagnosed among almost all age groups. The incidence of breast cancer increased significantly after 35 years of age, and stayed high between the ages 45-80. The incidences of colorectal, lung, and stomach cancers also increased significantly with age, and the sharp increase was seen after ages 50, 55, and 65, respectively.

3.2. *Cancer mortality*

The annual number of deaths, crude and age-standardized mortality for all cancers are presented in Table 3. A total of 8,331 patients died of cancer from January 2002 to December 2011. There were 4,694 (56.34%) cancer deaths of males, and 3,637 (43.66%) cancer deaths of females. The male to female ratio was 1.29:1. For all cancers combined, the crude mortality of males and females were 298.69 and 226.81 per 100,000 population, respectively. Age-standardized rates by the 2000 Chinese standard population were 118.82 and 73.78 per 100,000 population for males and females, and age-standardized rates by Segi's standard were 147.04 and 90.62 per 100,000 population for males and females, respectively. Male mortality for all cancers combined was significantly higher than the female mortality ($u = 12.52$, $p < 0.01$).

The number of cancer deaths and mortality for common cancer types by sites are presented in Table 4. The top 10 causes of cancer deaths among males were: lung, stomach, colorectal, liver, pancreas, prostate, esophageal, lymphoma, bladder, and leukemia, accounting for approximately 84 percent of all cancer deaths. Lung cancer alone was accounting for about one quarter of all cancer deaths in males. The corresponding cancers among females were: lung, colorectal, stomach, breast, liver, pancreas, gallbladder, ovary, lymphoma, and brain, accounting for about 78 percent of all cancer deaths. The mortality rates of lung ($u = 13.89$, $p < 0.01$), stomach ($u = 8.95$, $p < 0.01$), liver ($u = 11.38$, $p < 0.01$),

Table 2. Cancer cases, crude and adjusted incidences by sex in Luwan district of Shanghai, 2002-2011

Site	ICD-10	Males				Females			
		Cases	Incidence (10^5)	ASR China* (10^5)	ASR World† (10^5)	Cases	Incidence (10^5)	ASR China* (10^5)	ASR World† (10^5)
Lip, oral cavity, & pharynx (except nasopharynx)	C00-10, C12-14	94	5.98	3.13	3.54	59	3.68	1.77	2.12
Nasopharynx	C11	86	5.47	3.46	3.64	33	2.06	1.28	1.31
Esophagus	C15	209	13.30	5.73	7.13	76	4.74	1.34	1.74
Stomach	C16	862	54.85	23.36	28.56	510	31.80	11.90	13.81
Colon and rectum	C18-21	940	59.81	25.90	32.19	932	58.12	21.67	26.88
Liver	C22	610	38.82	19.14	21.88	265	16.53	5.48	6.78
Gallbladder	C23-24	94	5.98	2.22	2.89	175	10.91	3.30	4.15
Pancreas	C25	254	16.16	6.93	8.40	231	14.41	4.28	5.26
Larynx	C32	77	4.90	2.16	2.68	6	0.37	0.18	0.24
Lung	C33-34	1269	80.75	33.68	41.79	693	43.22	15.46	19.38
Other thoracic organs	C37-38	24	1.53	0.99	1.00	17	1.06	0.79	0.73
Bone	C40-41	26	1.65	1.05	1.14	20	1.25	1.10	1.20
Melanoma of skin	C43	11	0.70	0.32	0.43	12	0.75	0.43	0.44
Breast	C50	8	0.51	0.24	0.26	1221	76.14	41.27	45.06
Cervix	C53	--	--	--	--	142	8.86	6.70	6.19
Uterus	C54-55	--	--	--	--	194	12.10	6.05	6.86
Ovary	C56	--	--	--	--	236	14.72	8.80	9.59
Prostate	C61	447	28.44	9.91	12.91	--	--	--	--
Testis	C62	13	0.83	0.90	0.75	--	--	--	--
Kidney	C64-66,68	240	15.27	7.94	9.42	139	8.67	3.94	4.52
Bladder	C67	263	16.74	6.80	8.63	87	5.43	1.93	2.48
Brain, CNS	C70-72	155	9.86	6.68	7.27	193	12.04	6.31	7.18
Thyroid	C73	117	7.44	5.55	5.61	319	19.89	14.85	15.02
Lymphoma	C81-85, 88, 90, 96	203	12.92	6.71	8.04	173	10.79	5.15	5.80
Leukemia	C91-95	143	9.10	5.85	6.82	108	6.74	4.09	4.44
All other sites and unspecified	A_0	418	26.60	12.46	14.50	439	27.38	12.03	13.86
All sites	ALL	6563	417.62	191.11	229.47	6280	391.63	180.11	205.05

ASR, age-standardized rate; CNS, central nervous system; ICD-10, International Classification of Diseases, 10th revision. *Age-standardized rate by Chinese standard population based on the result of national census in 2000. †Age-standardized rate by the Segi's world standard population.

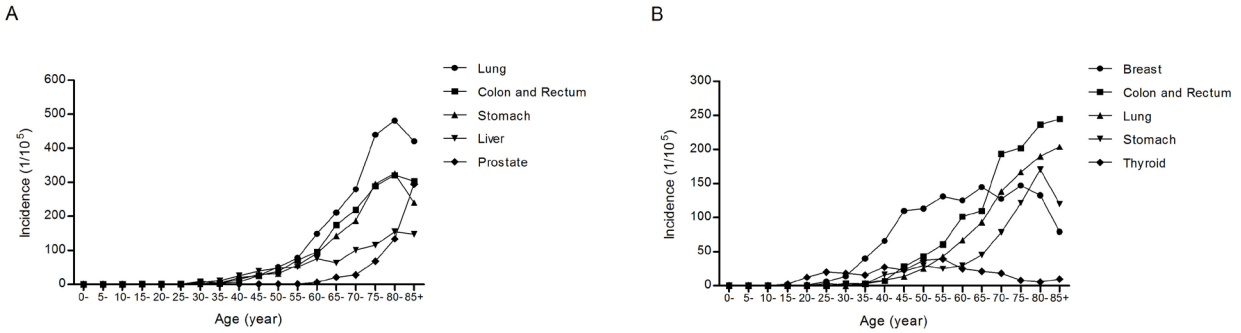

Figure 1. Age-specific incidences of the 5 most commonly diagnosed cancers among permanent residents in Luwan district of Shanghai, 2002-2011. (A) Age-specific incidences of the 5 most commonly diagnosed cancers among males in Luwan district of Shanghai, 2002-2011. **(B)** Age-specific incidences of the 5 most commonly diagnosed cancers among females in Luwan district of Shanghai, 2002-2011.

Table 3. Deaths and mortality of all malignant tumors in Luwan district of Shanghai, 2002-2011

Year	Males					Females					Total				
	Deaths	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)	Deaths	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)	Deaths	Population	Crude rate ($1/10^5$)	ASR China* ($1/10^5$)	ASR World† ($1/10^5$)
2002	505	169034	298.76	128.11	161.04	373	171089	218.02	80.03	97.05	878	340123	258.14	101.75	125.87
2003	463	164522	281.42	124.71	155.52	382	166582	229.32	78.22	98.15	845	331104	255.21	99.71	124.32
2004	467	162074	288.14	117.83	149.27	357	164138	217.50	77.42	91.89	824	326212	252.60	95.60	117.54
2005	440	159090	276.57	110.88	137.94	343	161257	212.70	74.82	88.38	783	320347	244.42	90.80	110.46
2006	442	156367	282.67	116.25	141.64	334	158819	210.30	68.99	85.50	776	315186	246.20	91.21	111.62
2007	428	154860	276.38	110.42	133.67	334	157770	211.70	68.54	83.16	762	312630	243.74	88.17	106.69
2008	474	153593	308.61	121.88	149.92	369	157235	234.68	76.06	93.15	843	310828	271.21	97.49	119.38
2009	501	152216	329.14	124.44	149.69	381	156526	243.41	78.54	98.24	882	308742	285.68	99.79	121.78
2010	504	150506	334.87	115.48	144.68	422	155387	271.58	73.15	93.79	926	305893	302.72	93.24	117.91
2011	470	149281	314.84	107.69	134.78	342	154752	221.00	61.89	76.60	812	304033	267.08	83.70	104.20
Total	4694	1571543	298.69	118.82	147.04	3637	1603555	226.81	73.78	90.62	8331	3175098	262.39	94.74	116.69

*Age-standardized rate by Chinese standard population based on the result of national census in 2000. †Age-standardized rate by the Segi's world standard population.

Table 4. Cancer deaths, crude and adjusted mortality rates by sex in Luwan district of Shanghai, 2002-2011

Site	ICD-10	Males				Females			
		Deaths	Mortality (10^5)	ASR China* (10^5)	ASR World† (10^5)	Deaths	Mortality (10^5)	ASR China* (10^5)	ASR World† (10^5)
Lip, oral cavity, & pharynx (except nasopharynx)	C00-10, C12-14	52	3.31	1.37	1.61	36	2.25	0.58	0.78
Nasopharynx	C11	52	3.31	1.72	2.08	17	1.06	0.38	0.47
Esophagus	C15	196	12.47	4.95	6.21	70	4.37	1.11	1.41
Stomach	C16	685	43.59	20.46	25.80	401	25.01	7.96	9.52
Colon and rectum	C18-21	546	34.74	13.03	16.47	558	34.80	10.24	12.98
Liver	C22	527	33.53	15.84	18.36	223	13.91	4.13	5.17
Gallbladder	C23-24	89	5.66	2.00	2.58	157	9.79	2.63	3.30
Pancreas	C25	230	14.64	6.11	7.36	219	13.66	3.91	4.93
Larynx	C32	47	2.99	0.97	1.29	6	0.37	0.16	0.24
Lung	C33-34	1178	74.96	28.87	36.12	609	37.98	11.92	15.01
Other thoracic organs	C37-38	19	1.21	0.50	0.71	12	0.75	0.47	0.45
Bone	C40-41	19	1.21	0.43	0.54	15	1.07	0.63	0.75
Melanoma of the skin	C43	5	0.32	0.12	0.16	2	0.12	0.03	0.10
Breast	C50	6	0.38	0.11	0.13	399	24.88	9.49	11.44
Cervix	C53	--	--	--	--	65	4.05	1.70	1.68
Uterus	C54-55	--	--	--	--	64	3.99	1.26	1.48
Ovary	C56	--	--	--	--	111	6.92	2.95	3.67
Prostate	C61	204	12.98	3.54	4.93	--	--	--	--
Testis	C62	2	0.13	0.13	0.11	--	--	--	--
Kidney	C64-66	65	4.14	1.42	1.79	60	3.74	1.15	1.44
Bladder	C67	137	8.72	2.29	3.27	51	3.18	0.60	0.82
Brain, CNS	C70-72	85	5.41	3.29	3.75	83	5.18	2.08	2.34
Thyroid	C73	11	0.70	0.29	0.37	18	1.12	0.32	0.44
Lymphoma	C81-85, 88, 90, 96	145	9.23	4.41	5.33	94	5.86	2.44	2.78
Leukemia	C91-95	112	7.13	4.24	4.96	72	4.49	2.14	2.51
All other sites and unspecified	A_0	282	17.94	6.86	8.36	295	18.40	5.52	6.93
All sites	ALL	4694	298.69	118.82	147.05	3637	226.81	73.78	90.62

ASR, age-standardized rate; CNS, central nervous system; ICD-10, International Classification of Diseases, 10th revision. *Age-standardized rate by Chinese standard population based on the result of national census in 2000. †Age-standardized rate by the Segi's world standard population.

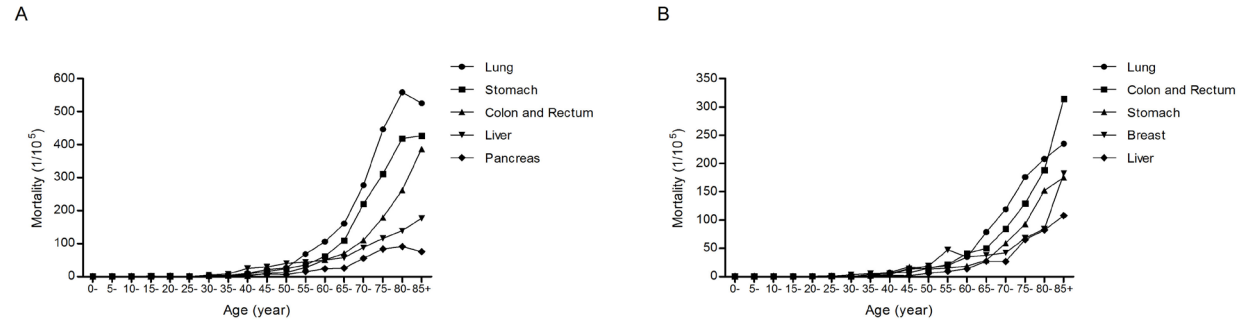

Figure 2. Age-specific mortality rates of the 5 most common causes of cancer deaths among permanent residents in Luwan district of Shanghai, 2002-2011. (A) Age-specific mortality rates of the 5 most common causes of cancer deaths among males in Luwan district of Shanghai, 2002-2011. **(B)** Age-specific mortality rates of the 5 most common causes of cancer deaths among females in Luwan district of Shanghai, 2002-2011.

Table 5. Death-to-case ratio by sex in Luwan district of Shanghai, 2002-2011

Year	Males			Females			Total		
	New cases	Deaths	Deaths /New cases	New cases	Deaths	Deaths /New cases	New cases	Deaths	Deaths /New cases
2002	654	505	0.77	582	373	0.66	1236	878	0.71
2003	605	463	0.77	585	382	0.65	1190	845	0.71
2004	658	467	0.71	572	357	0.62	1230	824	0.67
2005	586	440	0.75	607	343	0.57	1193	783	0.66
2006	666	442	0.66	592	334	0.56	1258	776	0.62
2007	631	428	0.68	615	334	0.54	1246	762	0.61
2008	641	474	0.74	635	369	0.58	1276	843	0.66
2009	681	501	0.74	601	381	0.63	1282	882	0.69
2010	719	504	0.70	738	422	0.57	1457	926	0.64
2011	722	470	0.65	753	342	0.45	1475	812	0.55
Total	5304	3726	0.70	5113	2882	0.56	10417	6608	0.63

esophageal ($u = 7.89$, $p < 0.01$), bladder ($u = 6.41$, $p < 0.01$) cancers, lymphoma ($u = 3.46$, $p < 0.01$) and leukemia ($u = 3.09$, $p < 0.01$) were significantly higher among males than females. While the mortality rate of gallbladder cancer ($u = 4.18$, $p < 0.01$) was significantly higher among females than males. There were no significant differences for colorectal ($u = 0.03$, $p > 0.05$), pancreas ($u = 0.73$, $p > 0.05$), brain ($u = 0.28$, $p > 0.05$), and kidney ($u = 0.56$, $p > 0.05$) cancers between males and females.

The age distributions of the top 5 causes of cancer deaths by sex are presented in Figure 2a and 2b. For both males and females, the mortality rates increased with age. Table 5 presents the death-to-case ration during the 10 years period.

3.3. *Trends in cancer incidence and mortality*

Trends in cancer incidence for all cancers combined and the top 10 most common cancer types are shown in Table 6. For both males and females, the age-standardized incidence rates increased significantly over the period of 2002 to 2011 ($p < 0.05$ for both). The temporal trend analyses among males showed incidences increased significantly of prostate and kidney cancers ($p < 0.05$ for both), while the incidence of stomach cancer decreased significantly ($p < 0.05$). The incidence trends for other selected cancer types in males were stable (Table 6). For females, the age-standardized incidence rate of breast cancer increased significantly from 2007 to 2011 ($p < 0.05$). A significant increase of age-standardized rate was also observed for thyroid cancer in females ($p < 0.05$). The temporal trends were stable for other selected cancer types in females (Table 6).

Table 7 shows trends in cancer mortality for all cancers combined and the top 10 most common cancer types. The age-standardized mortality rates stayed stable during the period of 2002 to 2011 for both males and females ($p > 0.05$ for both). The temporal trend analyses showed for males, the mortality rates of lung, stomach, and esophageal cancers decreased significantly ($p < 0.05$ for all three). The temporal trends of mortality for other selected cancer types in males were stable (Table 7). For females, the age-standardized mortality rate of lymphoma increased significantly from 2002 to 2011 ($p < 0.05$), and the temporal trends were stable for other selected cancer types (Table 7).

4. Discussion

The present study analyzed cancer composition, incidence, mortality and their trends among permanent

Table 6. Trends in cancer incidence rates (age-standardized to the Segi's world Standard Population) for selected cancers and all cancers combined by sex in Luwan district of Shanghai, 2002-2011

Site	ICD-10	Trends 1		Trends 2	
		Years	APC	Years	APC
Male					
Lung	C33-34	2002-2011	- 0.97		
Colon and rectum	C18-21	2002-2011	- 0.42		
Stomach	C16	2002-2011	- 3.33*		
Liver	C22	2002-2011	- 0.04		
Prostate	C61	2002-2011	6.42*		
Bladder	C67	2002-2011	1.03		
Pancreas	C25	2002-2011	2.96		
Kidney	C64-66,68	2002-2011	6.19*		
Esophagus	C15	2002-2011	- 6.24		
Lymphoma	C81-85, 88, 90, 96	2002-2011	3.22		
All sites	ALL	2002-2011	1.25*		
Female					
Breast	C50	2002-2007	- 3.31	2007-2011	10.48*
Colon and rectum	C18-21	2002-2011	0.49		
Lung	C33-34	2002-2011	1.29		
Stomach	C16	2002-2011	1.67		
Thyroid	C73	2002-2004	109.32*	2004-2011	18.28*
Liver	C22	2002-2011	0.40		
Ovary	C56	2002-2011	- 8.44		
Pancreas	C25	2002-2011	4.63		
Uterus	C54-55	2002-2011	9.56		
Brain, CNS	C70-C72	2002-2011	1.22		
All sites	ALL	2002-2011	3.06*		

APC, annual percent change; CNS, central nervous system; ICD-10, International Classification of Diseases, 10th revision. *The APC is significantly different from zero ($P < 0.05$).

Table 7. Trends in cancer mortality rates (age-standardized to the Segi's world Standard Population) for selected cancers and all cancers combined by sex in Luwan district of Shanghai, 2002-2011

Site	ICD-10	Trends 1		Trends 2		Trends 2	
		Years	APC	Years	APC	Years	APC
Male							
Lung	C33-34	2002-2011	- 3.12*				
Stomach	C16	2002-2011	- 3.34*				
Colon and rectum	C18-21	2002-2011	2.46				
Liver	C22	2002-2011	- 1.86				
Pancreas	C25	2002-2004	- 33.95	2004-2007	26.40	2007-2011	- 5.41
Prostate	C61	2002-2011	3.37				
Esophagus	C15	2002-2011	- 9.18*				
Lymphoma	C81-85, 88, 90, 96	2002-2011	6.63				
Bladder	C67	2002-2011	2.25				
Leukemia	C91-95	2002-2011	1.25				
All sites	ALL	2002-2011	- 0.83				
Female							
Lung	C33-34	2002-2004	- 23.17	2004-2007	14.6	2007-2011	- 12.26
Colon and rectum	C18-21	2002-2011	- 1.59				
Stomach	C16	2002-2011	- 2.42				
Breast	C50	2002-2011	- 0.71				
Liver	C22	2002-2011	- 0.75				
Pancreas	C25	2002-2011	- 0.41				
Gallbladder	C23-24	2002-2011	- 6.58				
Ovary	C56	2002-2011	- 1.33				
Lymphoma	C81-85, 88, 90, 96	2002-2011	21.20*				
Brain, CNS	C70-C72	2002-2011	8.13				
All sites	ALL	2002-2011	- 1.21				

APC, annual percent change; CNS, central nervous system; ICD-10, International Classification of Diseases, 10th revision. *The APC is significantly different from zero ($P < 0.05$).

residents in Luwan district of Shanghai from 2002 to 2011. A total of 12,843 new cancer cases were diagnosed during the period of 2002-2011, with a male to female ratio of 1.05:1. The crude incidences for all cancers combined were 417.62 and 391.63 per 100,000 population for males and females, respectively. The age-standardized incidences by Segi's standard population were 229.46 and 205.05 per 100,000 population for males and females, respectively. The age-standardized incidence rates of all cancers combined for both males and females increased significantly over the 10 year period, indicating the urgent needs of broadly applying effective prevention measures covering known cancer risk factors, especially for those cancer types with increasing temporal trends. From another standpoint, the high cancer incidence estimated in this developed district might be partly due to over-diagnosis of cancers by intense sensitive investigations in recent years. The total number of cancer deaths was 8,331, with a male to female ratio of 1.29:1. The crude mortality rates of all cancers combined were 298.69 and 226.81 per 100,000 population for males and females, respectively. The age-standardized mortality rates by Segi's standard population were 229.46 and 205.05 per 100,000 population for males and females, respectively. Certain cancer types, including lung, stomach, and esophageal cancers showed decreasing mortality trends in males, while the mortality of lymphoma showed a significant increasing trend in females.

The top 10 cancer types of Luwan district were different from the national statistics. Lung, stomach, liver, and esophageal cancers were the 4 most common cancer types diagnosed in China according to the cancer statistics of 2015 (*1*). Compared to the national data, lung and stomach cancers remained the most commonly diagnosed cancers in Luwan district. However, colorectal cancer (CRC) surpassed liver cancer and became the top diagnosed gastrointestinal cancer. Breast cancer was the fourth commonly diagnosed cancers and the most frequent one in females. In addition, esophageal cancer ranked 9th among males and did not make the top 10 list in females. This disparity reflected regional differences, which may be affected by many elements including changes of risk factors and detection techniques.

Lung cancer was the first and third most frequently diagnosed cancer in males and females, respectively. Similar to the national data, it was also the leading cause of cancer death among both males and females. In addition, the incidence trend of lung cancer during the 10 years period was stable, while the mortality rate decreased slightly in males. Worldwide, tobacco smoking is a major risk factor of cancer incidence and mortality including lung, stomach, pancreas, liver, kidney, urinary tract, and uterine cervix (*6*). With the high smoking rate in adult Chinese men, and still rising rates in adolescents and young adults, smoking-related cancer will continue to be a huge public health burden in China (*7,8*). Cigarette smoking is the single largest cause of lung cancer, accounting for about 90% of all diagnosed cases (*9*). For Chinese women, besides the traditional risk factors, environmental pollution such as passive smoking and cooking smog are also contributors of lung cancer (*10*). Public health campaigns and tobacco control programs to prevent initiation and promote cessation in some western countries have successfully decreased smoking rate, which preceded the decrease of lung cancer incidence and mortality rates especially among males (*11,12*). Similarly, we observed a decreasing trend of lung cancer mortality among male residents in Luwan district. However, like other areas in China (*1*), lung cancer has been and will remain to be a major health burden in Luwan district. Furthermore, most lung cancers are diagnosed at later stages, missing the best opportunity for effective treatment and resulting in poor prognosis (*13*); therefore, effective lung cancer screening, especially for high risk population is of crucial importance for improving survival and life quality. Studies have shown that compared with chest x-ray, annual screening for lung cancer with low-dose computed tomography (LDCT) significantly reduced lung cancer mortality (*14*). It is of great value to carry out such parallel screening studies in the communities of Shanghai.

A significant increase of incidence was seen for cancers of the prostate and kidney for men, and breast and thyroid for women. The exact reasons for the increase are not fully elucidated; however, westernized diet and physical inactivity may contribute to some of the changes (*1*). Improvements in several aspects, including elevation of disease awareness, completeness of the data, improvement of detection service, and gradual implementation of screening procedures such as prostate-specific antigen screening may also account for the marked increase (*1,15*). An increasing trend of breast cancer incidence was observed from 2007 to 2011, which may be partially influenced by reproductive changes in Chinese women (*16*). Breast cancer screening is well-implemented and common in the United States, while it is relatively new in China (*17,18*). Approaches or strategies for cancer prevention and early detection should be tailored according to the unique cultural beliefs of the population and the policies of the government. It is essential to raise awareness, especially in high-risk women, of the importance of breast cancer screening. Similar to the national statistics and study results from other countries (*1,19-21*), it is reported that there was a dramatic increase of thyroid cancer in women of Luwan district, and it is unclear whether the rise is due to "over-diagnosis" using new imaging technologies or is a real increase caused by change of exposure level to risk factors (*22,23*).

Major gastrointestinal cancers including cancers of esophagus, stomach, colon and rectum, liver, gallbladder, and pancreas accounted for approximately 45 percent

and 35 percent of the total new cancer cases in men and women respectively in Luwan district during the 10-year period. The high proportion of gastrointestinal cancers may reflect the changes of population characteristics such as high smoking rate, inactive life style, more meat and less fresh fruits and vegetables in diet. CRC ranked the second most common cancer type in both males and females, and has been continuously posing a threat to people's health. Regular screening has been shown to be effective in preventing CRC and reducing CRC mortality (*24*). The ongoing CRC screening program in Shanghai using fecal occult blood test has identified a sizeable proportion of high-risk individuals and is waiting for the long-term results (*25*).

Compared to other areas in China, the incidence of liver cancer in the district was relatively low, which can be attributed to the successful control of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, as well as effective implementation of the HBV vaccination program (*26*). A significantly decreasing incidence and mortality trend was observed for stomach cancer during the 10 year period in Luwan district. There was also a decreasing trend of esophageal cancer mortality. Improved sanitation and greater availability of fresh food are main contributors to the decline (*27,28*).

There are some limitations of the current study. Our dataset was from a single district in Shanghai, which was a typical urban area with well developed economy. Therefore, it may not be representative of metropolitan Shanghai. However, our study utilized complete and accurate data from the community, and population-based data are crucial to plan and assess the effectiveness of prevention and control strategies. In 2011, there was a revocation of the organizational system of Shanghai Luwan district and Huangpu district, and then the establishment of a new Huangpu district. The jurisdiction of the original Luwan district was adjusted to the new Huangpu district. Therefore, our study result is also of historical value.

In summary, the current study comprehensively analyzed community based cancer statistics including composition, incidence, mortality, and temporal trends, which provided valuable information for developing and evaluating cancer prevention and control strategies. The significance of cancer registration for disease surveillance and management should be brought into focus. With an aging population, cancer will continue to be a huge public health problem nationwide. It is important to further study the epidemiology and etiology of cancers so as to increase existing cancer control knowledge, reduce preventable cancers and ultimately relieve future cancer burden.

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Brief Report

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Estimation of lactic acid bacterial cell number by DNA quantification

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Summary **Lactic acid bacteria are provided by fermented foods, beverages, medicines, and supplements. Because the beneficial effects of medicines and supplements containing functional lactic acid bacteria are related to the bacterial cell number, it is important to establish a simple method for estimating the total number of lactic acid bacterial cells in the products for quality control. Almost all of the lactic acid bacteria in the products are dead, however, making it difficult to estimate the total number of lactic acid bacterial cells in the products using a standard colony-counting method. Here we estimated the total lactic acid bacterial cell number in samples containing dead bacteria by quantifying the DNA. The number of viable *Enterococcus faecalis* 0831-07 cells decreased to less than 1×10^{-8} by 15 min of heat treatment at 80°C. The amount of extracted DNA from heat-treated cells was 78% that of non-heated cells. The number of viable *Lactobacillus paraplantarum* 11-1 cells decreased to 1×10^{-4} after 4 days culture. The amount of extracted DNA of the long-cultured cells, however, was maintained at 97%. These results suggest that cell number of lactic acid bacteria killed by heat-treatment or long-term culture can be estimated by DNA quantification.**

Keywords: Lactic acid bacteria, medicines, supplements, DNA quantification, total cell number estimation

1. Introduction

Lactic acid bacteria have various functions, such as digestive functions and immunostimulation, which are considered to be beneficial for maintaining human health (*1*). Functional lactic acid bacteria are provided as fermented foods, such as yogurt, pickles, and fermented juices, as well as in medicines and supplements (*2,3*). We previously screened functional lactic acid bacteria using a unique evaluation system utilizing silkworms as experimental animals (*4-8*).

During the course of those studies, we realized that the development of a simple method for estimating the total number of lactic acid bacterial cells in samples is important for validation of foods and supplements containing functional lactic acid bacteria. The total number of lactic acid bacterial cells in samples is the sum of viable and dead cells. Measuring the number of viable cells is relatively easy by spreading appropriately diluted samples on agar plates, incubating the plates, and counting the resulting colonies. The number of viable cells is substantially decreased, however, by long-term culture or sterilization during the manufacturing the process. Therefore, a simple method for determining the total cell number of lactic acid bacteria is desired. The use of a dye that stains lactic acid bacteria (*9*) or an antibody against lactic acid bacteria was proposed for measuring the number of lactic acid bacteria (*10,11*). These methods, however, may be problematic, requiring special instruments and complicated techniques, and having limited application to specific lactic acid bacteria species.

DNA quantification has been proposed for estimating the total number of bacterial cells in samples containing both viable and dead cells (*12*). A possible problem in applying this method to lactic acid bacteria is a decrease in the amount of DNA due to its degradation during long-term culture or sterilization. In the present study, we show that the amount of DNA from lactic acid bacteria is still maintained under conditions in which viable cells were decreased. Our results suggest that the total bacterial number of lactic acid bacteria can be estimated

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by DNA quantification.

2. Materials and Methods

2.1. *Lactic acid bacteria used in this study*

Leuconostoc paraplantarum 11-1 (*4*) and *Enterococcus faecalis* 0831-07 are the lactic acid bacteria isolated in our laboratory.

2.2. *Preparation of lactic acid bacteria*

Glycerol stocks of lactic acid bacteria kept at –80°C were thawed at room temperature and spread on MRS (de Man, Rogosa, Sharpe) agar plates, followed by culture at 30°C. The emerged colonies were cultured in MRS medium at 30°C in a bottle. Cells were heat-treated by placing full-growth cell cultures at 80°C for 15 min.

2.3. *Measurement of viable cell number*

A 100-µL aliquot of lactic acid bacteria culture was diluted in saline and spread on an MRS agar plate. After incubation at 30°C, the number of colonies was counted, and number of viable cells in the sample was calculated.

2.4. *DNA quantification*

Bacterial cells were harvested from 1.5 mL of culture, washed with 1 mL saline, and pelleted by centrifugation, and then the cell pellets were suspended in 0.6 mL of DNA extraction buffer (20 mM Tris-HCl (pH 8.0), 25 mM EDTA, 250 mM NaCl, 1% SDS). The suspension was mixed with 0.5 mL zirconium oxide beads (Yasui Kikai Corporation, Osaka, Japan) and the cells were homogenized using a bead-type homogenizer (Yasui Kikai Corporation, Osaka, Japan) at 2,500 rpm for 5 min. The samples were mixed with 0.5 mL phenol/chloroform/isoamyl alcohol, shaken vigorously, and centrifuged at 12,000 rpm for 5 min. The aqueous layer was collected and washed with 0.5 mL of chloroform, followed by ethanol precipitation. Precipitates were dissolved in 100 µL Tris-EDTA buffer and treated with 50 µg/mL RNaseA (NIPPON GENE CO., LTD, Toyama, Japan). Samples were extracted with phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. The sample was dissolved in 100 µL of Tris-EDTA buffer. The amount of DNA was determined by fluorometric determination (Qubit™ dsDNA HS Assay Kit, Invitrogen) based on the detection of DNA-specific fluorescence.

3. Results

3.1. *Relationship between amount of DNA and viable cell number of exponentially growing cells*

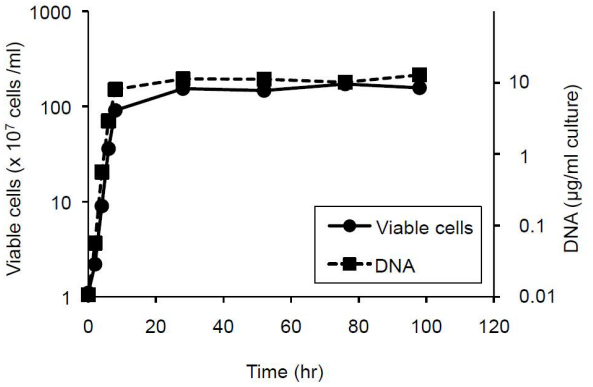

Figure 1. Time-course of the changes in colony-forming units and amount of DNA of lactic acid bacteria *Enterococcus faecalis* 0831-07. *E. faecalis* 0831-07 was cultured in MRS liquid media and sampled periodically. Viable bacterial number was calculated by counting the colonies on agar plates on which appropriately diluted samples had been spread and incubated. The amounts of DNA extracted from the bacterial samples were determined by a fluorometric method as described in the Materials and Methods section.

We cultured an *Enterococcus faecalis* strain (0831-07) in liquid medium and periodically sampled the culture over 5 days. DNA was extracted from the bacterial samples and quantified as described in the Materials and Methods section. During the exponentially growing phase (4-8 h), the amount of DNA increased along with the number of viable cells (Figure 1). At the stationary phase, in which the number of viable bacteria did not increase, the amount of DNA also did not increase (Figure 1). The ratios between the amount of DNA (µg/mL•culture) and the number of viable cells ($\times 10^7$ cells/mL) were almost constant (0.059-0.088) for 4 to 100 h after inoculation. Therefore, when lactic acid bacteria cells proliferate exponentially or even at the stationary phase, it is possible to predict the number of cells on the basis of the amount of DNA.

3.2. *Quantification of DNA in heat-treated lactic acid bacteria*

In most cases, the lactic acid bacteria used for supplements is killed due to heat-treatment or long-term culture during the production process. Therefore, under these conditions, the total number of bacterial cells in the samples is much different from the viable number of cells. The number of viable *E. faecalis* 0831-07 cells cultured at 30°C for 28 h was 1.6×10^9 cfu/mL. After treatment at 80°C for 15 min, the number of viable cells decreased to less than 10 cfu/mL (Figure 2A). The amount of DNA extracted from the heat-treated cells was 78% that extracted from non-heat-treated samples (Figure 2B). Heat treatment at 80°C for 15 min remarkably decreased the number of viable cells, whereas the amount of DNA remained at the same level as before the heat treatment. The size of chromosomal DNA extracted from the heat-treated

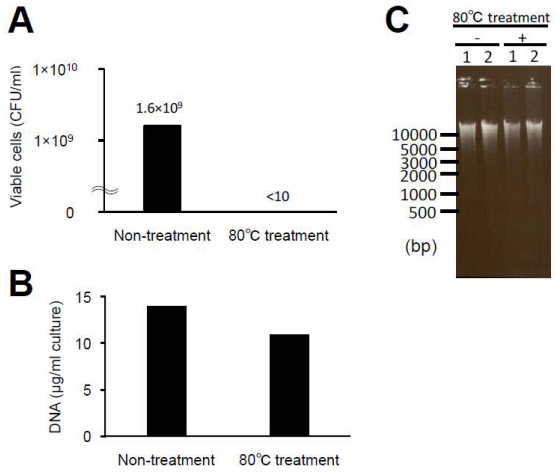

Figure 2. Changes in colony-forming units and amount of DNA after heat-treatment of *E. faecalis* 0831-07. *E. faecalis* 0831-07 was cultured in MRS liquid media for 28 h and heat-treated at 80°C for 15 min. Viable bacterial cell number per milliliter of culture media was calculated by counting the colonies on agar plates on which the samples had been spread and incubated **(A)**. DNA was extracted from the sample and analyzed by DNA quantification **(B)** or agarose gel electrophoresis **(C)**.

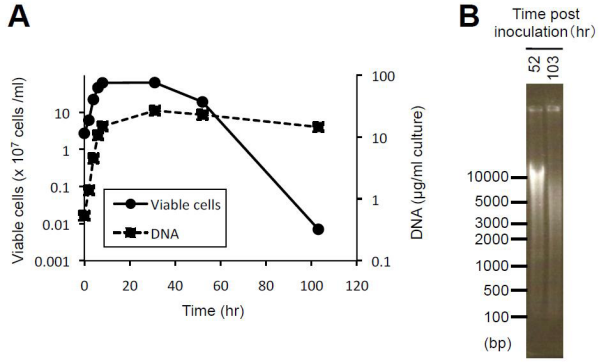

Figure 3. Time-course of the changes of colony-forming units of lactic acid bacteria *Lactobacillus paraplantarum* 11-1, amount of DNA, and degradation of DNA after long-term culture. *Lactobacillus paraplantarum* 11-1 was cultured in MRS liquid media and sampled periodically. Viable bacterial cell number was calculated by counting the colonies on agar plates on which the samples had been spread and incubated. The amount of DNA extracted from the bacterial samples was determined by a fluorometric method **(A)**. DNA extracted from *L. paraplantarum* 11-1 was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Photography was taken on a UV transilluminator **(B)**.

sample was indistinguishable from that extracted from the non-heat-treated samples when analyzed by agarose gel electrophoresis (Figure 2C), suggesting that the heat treatment did not degrade the DNA.

3.3. *The amount of DNA in lactic acid bacterial cells after long-term culture*

L. paraplantarum 11-1 is a lactic acid bacterium with high innate immunity stimulating activity (*4*). This bacterium is useful for manufacturing of healthy foods. When overnight culture of *L. paraplantarum* 11-1 in stationary phase was cultured for an additional 4 days, the number of viable bacteria decreased to 1/10,000 that of the stationary phase culture (Figure 3A). Analysis by agarose gel electrophoresis revealed that the DNA in the long-term cultured *L. paraplantarum* 11-1 degraded into small fragments (Figure 3B). The amount of DNA from the long-term cultured *L. paraplantarum* 11-1, however, was 97% that of the stationary phase culture (Figure 1). This indicates that the number of the heat-killed lactic acid bacteria can be estimated by quantifying the DNA.

3.4. *Estimation of total cell number of lactic acid bacteria by DNA determination*

We next attempted to estimate the total number of lactic acid bacterial cells from the amount of DNA extracted from the cells (Table 1). The sizes of the *E. faecalis* and *L. paraplantarum* genomes are 3.2 and 3.1 Mbp, respectively, which means the amount of one genome of DNA is 3.3 fg and 3.2 fg, respectively (*13,14*) (Table 1). The concentration of the DNA harvested from overnight (28 h, stationary phase) cultures of *E. faecalis* and *L. paraplantarum* was 11 and 27 μg/mL of culture, respectively. The ratio of the total cell number calculated from the DNA amount and the viable cell count was 2.3 and 13, respectively (Table 1). The cell number calculated from the DNA amount was much larger than the viable cell number calculated from the colony count. This is probably caused by the presence of unculturable bacteria (including dead cells) in the samples. Therefore, it is important to note that the total

Table 1. Total cell number of *Enterococcus faecalis* 0831-07 and *Lactobacillus paraplantarum* #11-1 cells estimated by DNA quantification

Species	Strain	Genome size (Mbp)	Calculated amount of genome DNA/cell (fg)	Determined amount of DNA (μg/mL culture)	Cell number calculated from DNA amount (× 10^9 CFU/ml culture)	Viable cell number (× 10^9 CFU/mL culture)	Cell number calculated from DNA amount/Viable cell number
Enterococcus faecalis	0831-07	3.2	3.3	11	3.4	1.5	2.3
Lactobacillus paraplantarum	#11-1	3.1	3.2	27	8.3	0.63	13

The cells were cultured in MRS medium at 30°C for 28 h. The amount of genome DNA in cells was calculated from the genome sizes of *E. faecalis* and *L. paraplantarum*. The DNA amounts were determined as described in the Materials and Methods section. Molecular weight of one pair of deoxynucleotides is 616. Avogadro's constant is 6.02×10^{23}.

cell number estimated by DNA quantification is always much larger than that of viable cells.

4. Conclusion

In the present study, we showed that the amount of DNA in lactic acid bacteria was maintained at a certain level, even though almost of the cells were killed by heat-treatment or by long-term culture. This indicates that the number of lactic acid bacteria can be estimated on the basis of the amount of DNA in fermented foods or supplements.

Acknowledgements

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Brief Report

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1,2,3-Triazolyl ester of ketorolac (15K): Boosting both heat-endurance and lifespan of *C. elegans* by down-regulating PAK1 at nM levels

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Summary **PAK1 (RAC/CDC42-activated kinase 1) is the major oncogenic/ageing kinase, and its dysfunction extends the healthy lifespan of *C. elegans* by activating *HSP16* gene. 15K is a highly cell-permeable 1,2,3-triazolyl ester of ketorolac that down-regulates both PAK1 and its down-stream COX-2 in *R*- and *S*-forms, respectively. 15K is 500-5,000 times more potent than ketorolac, an old pain-killer, inhibiting the growth of cancer cell lines with IC_{50} ranging 5-24 nM. Scores of natural and synthetic PAK1-blockers have been shown to extend the lifespan of small animals such as *C. elegans*, but none of them has been effective at nM levels. Thus, we examined *in vivo* effect of 15K at nM levels on the survival rate of *C. elegans* with or without heat-shock. Like the PAK1-deficient mutant, 15K (at 50 nM)-treated worm significantly lives longer, is far more heat-resistant and less productive (fertile) than the non-treated counterpart, with an increased expression of *HSP16* gene. 15K has been proven to be among the most potent anti-cancerous and longevity-promoting PAK1-blockers, and therefore has a potential to treat a variety of solid tumours without severe side effect.**

Keywords: Ketorolac, 1,2,3-triazolyl ester, PAK1, *C. elegans*, lifespan, anti-cancer

1. Introduction

PAK1 is a Ser/Thr-kinase that is activated by the GTPases (RAC/CDC42), and essential for the growth of over 30% human cancer called RAS cancers which carry the oncogenic mutant of Ki-RAS (*1-3*). This "RAS cancer" represent over 90% of pancreatic cancer, 50% of colon cancer and 30% of lung cancer (*1*). Furthermore, since solid tumours require PAK1-dependent angiogenesis for their robust growth (*2-4*), it is most likely that the majority of other solid tumours also depends on PAK1. Interestingly PAK1 is not essential for normal cell growth (*2,3*). Furthermore, PAK1-deficient mutant (RB689) of *C. elegans* lives 60% longer than the wild-type, clearly indicating that PAK1 is among the major ageing kinases shortening the healthy lifespan (*5*). Thus, in principle, unlike all conventional anti-cancer drugs (DNA/RNA/microtubules poisons), none of highly-specific PAK1-blockers are expected to cause any severe side effects. In fact scores of natural PAK1-blockers and even a few synthetic ones have been shown to extend the lifespan of small animals such as *C. elegans*, but so far none of them has been effective at nM levels (*6*).

We recently developed a potent PAK1-blocker called 15K from Ketorolac, an old pain-killer *via* Click Chemistry (*7*). 15K is a highly cell-permeable 1,2,3-triazolyl ester of ketorolac (Figure 1), a racemic COOH-bearing PAK1-blocker/COX-2 inhibitor, which down-regulates PAK1 in *R*-form, as well as directly inhibits COX-2 in *S*-form (*7,8*). 15K is over 500-5,000 times more potent than ketorolac to inhibit the growth of cancer cells such as A549 (lung) and B16F10 (melanoma) cell lines with IC_{50} ranging 5-24 nM (*7*).

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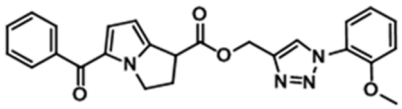

Figure 1. Chemical structure of 15K.

Furthermore, 15K inhibits the embryonic angiogenesis *in ovo* (fertilized eggs) with IC_{50} around 1 nmol/egg (*9*).

In this study, prior to *in vivo* (human cancer xenograft in mice) test of 15K, we examined whether 15K (10-100 nM) could extend (or shorten) the healthy lifespan of *C. elegans* which has the shortest mean lifespan (around 2 weeks) among experimental animals, as did either the PAK1-KO (knock-out) or a herbal PAK1-blocker called triptolide (around 140 μM) previously (*4,10*), in an attempt to confirm that 15K causes little side effect on this worm at least.

2. Materials and Methods

2.1. *Strain of C. elegans and reagents*

The strain CL2070 of *C. elegans* was kindly provided by CGC (*C. elegans* Genomic Center). 15K was synthesized from ketorolac *via* Click Chemistry as previously described (*7*).

2.2. *Measurement of brood size*

The wild-type (N2) of *C. elegans* was fed by the lawn culture of *E. coli* (OP50) which was grown in the presence or absence of 15K at 25, 50 and 100 nM on the standard NGM (nematode growth medium) agar plate for 2 days shortly after the hatching at 23°C. Then the number of eggs laid by each group of around 40 adult worms overnight (for 10 hours) was counted. The brood size was calculated as the number of eggs per mother (female worm).

2.3. *Survival of worms after prolonged heat-shock treatment*

The wild-type (N2) was fed by *E. coli* which was grown in the presence or absence of 10-100 nM of 15K for 2 days as described above. Then each group of 60 adult worms was heat-shocked at 35°C for 8 hours. Then each group was cultured at 20°C for over 2 weeks. Every day after the heat challenge, the number of "dead" worms in each group was counted for scoring their survival rate. For statistical analysis, we employed the log-rank analysis (*11*).

2.4. *HSP16.2 dependent GFP expression*

The strain CL2070 which carries a transgenic reporter gene called "HSP16.2-GFP" fusion gene (*12*) was fed by *E. coli* (OP50) which was gown in the presence or absence of 25-100 nM of 15K overnight (12 hours) at 22°C. Then each group of around 20 worms was heat-shocked at 35°C for 2 hours, and then kept at 22°C for the recovery. After 4 hours, each group was fixed with a drop (10 micro liter) of sodium azide (1 M) on slides for microscopy. Under blue light which stimulates the green florescence emission from GFP produced in each worm, the fluorescence images were acquired at the same exposure parameters, using a 40X objective of the microscope (BX60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; QImaging, Burnaby, British Columbia, Canada).

2.5. *Measurement of lifespan*

Five L4/young adult worms were transferred to a fresh NGM plate and permitted to lay eggs for 5 hours. After removing the five adult worms, the progeny were grown on NGM plates for 3 days. The lifespan of the age-synchronized hermaphrodites (*5,10,13*) at 20°C was measured on the agar plates with the lawn culture of the control *E. coli* (OP50) or those cultured with 15K at 10-100 nM, as we have done with triptolide (140 μM) previously (*13*). In order to prevent progeny production, 5-fluoro-2'-deoxyuridine (FUdR; Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the agar plate at the final concentration of 36 μM after the animals had reached adulthood as described previously (*5,10,13*). The log-rank test was employed for statistical analysis (*11*).

2.6. *Statistic analysis*

Data are expressed as mean values with their standard errors. Statistical comparisons were performed by one-way ANOVA. Statistical analysis was conducted using SPSS (release 16.0, Chicago, Illinois) and $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. *15K reduces the brood size (number of eggs laid) of C. elegans*

In the past, either PAK1-KO (knock-out) or treatment with herbal PAK1-blockers such as CAPE (caffeic acid phenethyl ester) reduced number of eggs laid by female worms, while it extended their healthy lifespan, indicating that there is a clear "trade-off" relationship between fertility and lifespan (*2,5*). Since fertility assay takes only a few days, while lifespan assay takes over a month, we first tested the effect of 15K on the number of eggs laid, in order to determine the effective doses of 15K. As shown in Figure 2, 15K at 50-100 nM reduced their fertility by 60-70% in a dose-dependent manner.

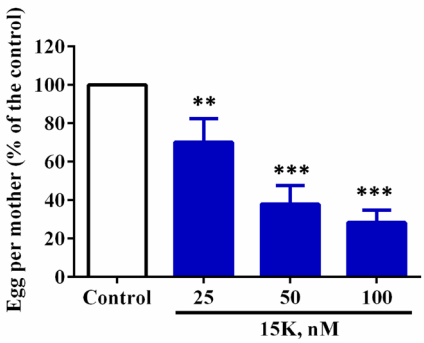

Figure 2. Reduction of brood size by 15K. Worms were pretreated with 15K at 25-100 nM, and the number of eggs laid by adult females was counted during 8 hrs. The brood size was reduced by 15K in a dose dependent manner. The results are mean ± SE of three independent experiments with 9 replicates per each treatment ($p \leq 0.01$). Statistically significant differences relative to the control are indicated by asterisks. $^{**}p < 0.01$, $^{***}p \leq 0.001$.

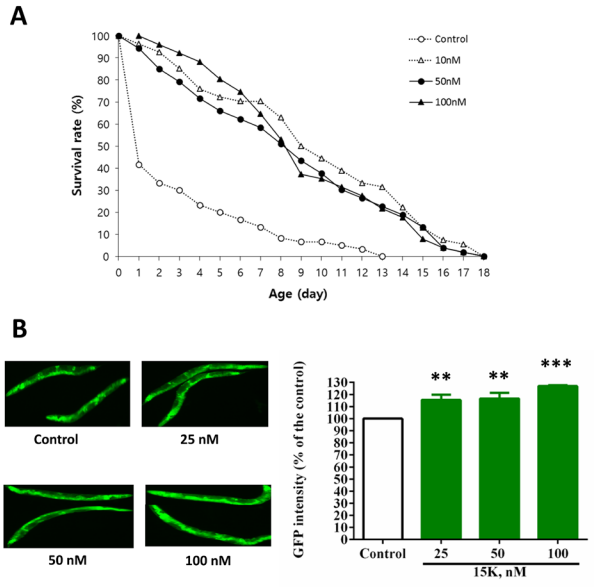

Figure 3. 15K-induced heat-resistance (A) by activating HSP16.2 gene (B). A. Heat-resistance. 15K (10-100 nM) treated *C. elegans* (closed circles or triangles) survived several times longer than the control worm (open circles) after heat-shock at 35°C for 8 hrs ($p \leq 0.001$). **B.** HSP16.2 gene activation. **Left side:** Image of HSP16.2:GFP expression in the control worms and those treated with indicated concentrations of 15K after heat-shock at 35°C for 2 hrs. **Right side:** GFP intensities were quantified in each CL2070 group. Each value represents the mean ± SE of four replicates (12 determinations) per each treatment. Statistically significant differences relative to the control are indicated by asterisks. $^{**}p < 0.01$, $^{***}p \leq 0.001$.

3.2. *15K increases the heat-resistance in C. elegans*

In the past, both reduction of their brood size and extension of their lifespan by down-regulation of PAK1 are closely associated with an increase in their heat-resistance and expression of a heat shock gene called HSP16.2 (*2,5*). Thus, we examined the effect of 15K on both heat-resistance and *HSP16.2* gene expression in CL2070 strain of *C. elegans*, where GFP is expressed under the control of *HSP16.2* gene promoter which is

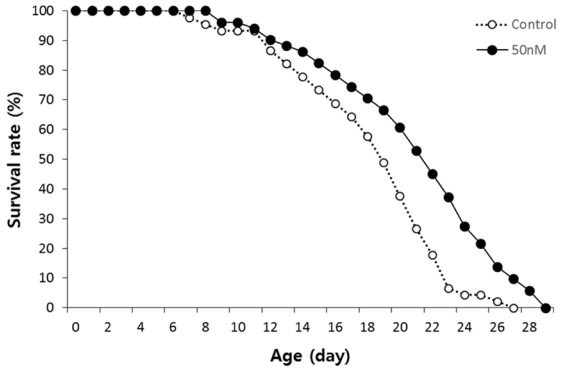

Figure 4. 15K extends the lifespan of C. elegans. Survival of age-synchronized worms was compared between untreated control (open circles) and 15K (50 nM)-treated worms (closed circles) at 20°C. At least the mean lifespan was clearly extended with 15K treatment ($p < 0.05$).

suppressed by PAK1 (*2,5,12*). As shown in Figure 3A, 15K (10-100 nM) treatment of this worm increased its heat-resistance (survival rate after heat-shock at 35°C for 8 hrs) by several times. As expected, GFP expression under the control *HSP16.2* gene promoter was also up-regulated with 15K (25-100 nM) treatment by 20-30% (see Figure 3B) after a brief (2 hrs) heat shock. Without heat-shock, however, no GFP was expressed (data not shown) as shown previously (*5,12*).

3.3. *15K extends the health lifespan of C. elegans*

The mean lifespan of the control worm is around 18 days as shown in Figure 4 (open circles). However, 15K treatment of this worm at 50 nM clearly increased the heathy lifespan to around 21 days by around 15% (see Figure 4, closed circles), as does the natural PAK1-blocker "triptolide" (140 μM) treatment (*13*). However, 10 nM 15K showed no effect on the mean lifespan of this worm (data not shown).

As mentioned previously (*7,8*), racemic 15K has at least two direct targets: (*i*) *R*-form directly inhibits the GTPase RAC that activates PAK1, and (*ii*) *S*-from directly inhibits COX-2 whose expression depends on PAK1. Our present test concerning the effect of 15K on *C. elegans* is basically same as phenotypes of PAK1 KO (*5*) as well as effect of natural PAK1-blockers such as propolis and triptolide (*13,14*), confirming that the down-regulation of both PAK1 and COX-2 by 15K leads to a significant extension, instead of shortening, of its lifespan.

The lifespan-extending effect of "synthetic" chemicals has been very rarely tested on *C. elegans*, although so many "natural" PAK1-blockers were tested with clearly positive outcome (*6*). The most likely reason appears to be at least in part due to a "myth" or "chemo-phobia" that, unlike natural compounds, synthetic chemicals in general would be rather harmful to living things. However, there is an exception:

metformin, an old synthetic anti-diabetic/anti-obesity compound was shown to extend the lifespan of *C. elegans* at 50 mM (*15*). Here in this study, we have presented a second exception with 15K, and the effective dose of 15K is 1 million times lower than that of metformin. Even compared with potent natural PAK1-blocking elixirs such as CAPE (ED = 100 μM) and triptolide (ED = 140 μM), 15K is over 1,000 times more potent as an elixir (longevity-promoter) than these natural PAK1-blockers (*6,13-16*).

In this context, we would like to encourage molecular oncologists or chemotherapists working on synthetic PAK1-blockers/inhibitors to test their lifespan-extending effect on short-lived tiny animals such as *C. elegans* and *Drosophila*, prior to either *in vivo* cancer xenograft in mice or clinical trials for cancer therapy, making it sure that each synthetic chemical causes no harm to these tiny invertebrates at least.

Very recently a Chinese team reported that even an old semi-synthetic pain-killer called "Aspirin" at 100 μM also extends the lifespan of this worm in association with an increased heat-resistance (*17*). Furthermore, a new synthetic chemical called NP1 that causes diet restriction also extends the lifespan of this worm at 50 μM (*18*). Thus, one could easily anticipate that even Ketorolac, an old synthetic pain-killer, which is 500 times less potent than 15K, also might extend the healthy lifespan of this worm at around 25 μM, although so far nobody appears to have tested its "elixir" (longevity-promoting) potential as yet.

It should also be worth pointing out that our assay for protection by 15K against pre-mature death of this worm after the prolonged heat-shock (equivalent to its protection against "global warming" effect) appears to be far more sensitive (and time-saving) than the standard assay for its life extending effect without heat-shock (compare Figure 3A and Figure 4). Thus, the former assay would be the better option (than the latter) for *in vivo* screening for PAK1-blockers such as 15K.

Lastly, since unlike vertebrates such as mice, *C. elegans* is among invertebrates that lack cardiovascular system, it would be worth testing the longevity-promoting effect of 15K in the shortest-lived vertebrate called African turquoise killifish as well, whose mean lifespan (around 4 months) could be extended by 50% by a natural PAK1-blocker called resveratrol at 2.5 μM (*19*). In general, up-regulation of PAK1 causes both hypertension and cranial hemorrhage in vertebrates such as zebra fish, and its down-regulation rescues the hypertension and hemorrhage (*2,20*), in favour of their longevity.

In conclusion, 15K is among the most potent synthetic PAK1-blockers as well as longevity-promoters, and it is most likely that 15K could be useful for treating a variety of solid tumors including neurofibromatosis (NF) as well as a series of many other PAK1-dependent diseases/disorders such as Alzheimer's disease (AD) and hypertension without severe side effect. We are currently testing this notion *in vivo* (in mouse models), prior to clinical trials.

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Brief Report

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Nosocomial pneumonia: Search for an empiric and effective antibiotic regimen in high burden tertiary care centre

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Summary **The clinical practice guidelines on nosocomial pneumonia recommends an empirical regimen that would work in 95% of the patients based on the local antibiogram. The aim of the study was development of an antibiogram for guiding empiric therapy in settings with high prevalence of multi-drug resistant organisms. A retrospective review of electronic health records (e-hospital portal) was done to analyze all respiratory isolates from patients admitted in medical wards and intensive care unit between May 2016 and May 2017. The samples included brocho-alveolar lavage (BAL), mini broncho-alveolar lavage (mini-BAL) and endotracheal aspirate. The sensitivity pattern (combined and individual) of all bacterial isolates were analysed for commonly used antibiotics and their combinations. Out of the 269 isolates, the most common organisms were *Pseudomonas aeruginosa* (125, 46%), *Acinetobacter baumanni* (74, 27%) and *Klebsiella pneumoniae* (50, 19%). Cefoperazone-sulbactam (43%) had the best sensitivity pattern overall. Cefoperazone-sulbactam plus amikacin (56%) was the combination with the best combined sensitivity overall. There is a high prevalence of resistance in the commonly implicated organisms to the available antibiotics. There is an urgent need for implementation of effective anti-microbial stewardship programmes and development of newer antimicrobials.**

Keywords: Hospital acquired pneumonia, ventilator associated pneumonia, polymyxins

1. Introduction

Ventilator-associated pneumonia (VAP) is defined as a pneumonia that develops at least after 48 hours of mechanical ventilation in an intubated patient (*1*). Hospital acquired pneumonia (HAP) develops only after 48 hours of admission in a non-intubated patient (*2*). The clinical practice guidelines on hospital acquired and ventilator associated pneumonia (VAP) by Infectious Disease Society of America (IDSA) recommends generation of local antibiograms to guide the optimal choice of empiric antibiotics (*3*). The regimen should be chosen in such a way that it should work in more than 95% of the patients (*3*). A single anti-pseudomonal that covers methicillin sensitive *Staphyloccus aureus* (MSSA) should be chosen in the empiric regimen. In patients with risk factors for multi-drug resistant organisms (MDR), two anti-pseudomonals of different classes should be used. A methicillin resistant *Stapylococcus aureus* (MRSA) coverage should be added when the patient has received prior antibiotics in the last 90 days or if > 20% of *S. aureus* isolates are methicillin resistant (*3*). The anti-pseudomonals that are used in empiric therapy include betalactams like piperacillin tazobactam (P/T), cefoperazone sulbactam (C/S) and carbapenems. The second anti-pseudomonal that is usually added is either a fluoroquinolone (FQ) or an aminoglycoside (AG). The problem in Indian settings is high prevalence of resistance limiting the number of antibiotics available for clinical use (*4,5*). The aim of the study was to, therefore, generate a local antibiogram for nosocomial

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pneumonia and choose an empiric regimen that would meet the above mentioned requirements.

2. Materials

A retrospective review of electronic health records (e-hospital portal) was done to analyze all the bacterial isolates from brocho-alveolar lavage (BAL), mini broncho-alveolar lavage (mini-BAL) and endotracheal aspirates. These were routine diagnostic samples collected from patients with suspected nosocomial pneumonia admitted in medicine wards and the medicine intensive care unit between May 2016 and May 2017. Non-duplicative significant isolates from each patient was included in the analysis. The samples were processed on a blood agar and a Mac Conkey agar plate. After 24 hours of incubation, they were only processed further if the colony count was > 10^5 CFU/mL for endotracheal aspirate and > 10^3 for bronch-alveolar lavage samples. The microbiologically significant isolates were further identified using conventional biochemicals. The antibiotic susceptibility was done using the Kirby Bauer disc diffusion method on a Muller Huenton agar. The isolates were deemed sensitive and resistant based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2016 (*6*). Following antibiotics were tested- piperacillin-tazobactam, amikacin, cefoperazone/sulbactam, ciprofloxacin, meropenem, imipenem, amoxicillin-clavulanic acid, cefotaxime and ceftazidime. The results of sensitivity were analysed using the WHONET software (*www.whonet.org/software.html*). The combined empiric sensitivity of all gram-negative organisms was also analysed. The sensitivity of commonly used combinations *i.e*. betalactams and a FQ or an AG were analysed. Sensitivity to either of the antibiotic in the combination was considered as sensitive for the combination. The sensitivity pattern of different antibiotics and combinations were analysed individually for isolates of *Acinetobacter baumanni*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

The frequency of sensitive and resistant strains was calculated as percentages with 95% confidence interval using the WHONET software. This was a retrospective review of electronic records and all care was taken to maintain the confidentiality of the patient details.

3. Results and Discussion

Out of the 269 isolates (Wards-155, ICU-114), the most common organism was *Pseudomonas aeruginosa* (125, 46%) followed by *Acinetobacter baumanni* (74, 27%), *Klebsiella pneumoniae* (50, 19%), *Escherichia coli* (10, 4%), *Enterobacter spp*. (5, 2%), *Burkholderia cepacia* (3, 1%) and *Staphylococcus aureus* (2, 1%). Cefoperazone-sulbactam (43%) was the antibiotic with the best overall sensitivity (Table 1). The antibiotic combination with the best sensitivity overall was found to be cefoperazone-sulbactam plus amikacin (56%) (Table 1). The antibiotic (individual) and antibiotic combination with the best sensitivity for *Acinetobacter baumanni* were cefoperazone sulbactam (50%) and cefoperazone sulbactam plus ciprofloxacin (54%) respectively (Table 2). The best sensitivity for *Klebsiella pneumoniae* was seen with imipenem (44%) while the best combination was imipenem plus amikacin (50%) (Table 2). The best sensitivity for *Pseudomonas aeruginosa* was seen with amikacin (59%) followed by meropenem (51%) while the best combination was meropenem plus amikacin (72%) (Table 2).

Table 1. Sensitivity pattern of all isolates of nosocomial pneumonia

Antibiotic name	%R	%S	%R, 95% CI
Piperacillin/Tazobactam	65.2	34.8	59.1 - 70.9
Amikacin	64	36	57.9 - 69.7
Cefoperazone/Sulbactam	57.2	42.8	51.0 - 63.2
Ciprofloxacin	74.6	25.4	68.8 - 79.6
Meropenem	64.8	35.2	58.7 - 70.5
Imipenem	60.6	39.4	54.4 - 66.5
Amoxicillin/Clavulanic acid	97.7	2.3	94.8 - 99.1
Cefotaxime	96.2	3.8	92.9 - 98.1
Ceftazidime	70.1	29.9	64.1 - 75.5
Cefoperazone/Sulbactam + Amikacin	43.6	56.4	37.6 - 49.8
Cefoperazone/Sulbactam + Ciprofloxacin	51.5	48.5	45.3 - 57.6
Imepenem + Ciprofloxacin	49.6	50.4	43.4 - 55.8
Imipenem + Amikacin	46.6	53.4	40.5 - 52.8
Meropenem + Ciprofloxacin	62.5	37.5	56.3 - 68.3
Meropenem + Amikacin	52.7	47.3	46.5 - 58.8
Piperacillin/Tazobactam + Amikacin	51.1	48.9	44.9 - 57.3
Piperacillin/Tazobactam + Ciprofloxacin	61	39	54.8 - 66.9

%R, percentage of resistant isolates. %S, percentage of sensitive isolates. CI, confidence interval.

The epidemiology of Indian critical care settings are different compared to western settings where gram positive organisms are the major concern (*7*). In a study by Gupta *et al*., it was found that gram negative organisms were more frequently associated with VAP while *Staphylococcus aureus* was associated with VAP in only 1.5% of cases (*8*). The most common organisms were *Acinetobacter spp*. followed by *Pseudomonas spp*. and *Escherichia coli* (*8*). In our study, almost all isolates were gram negative organisms with the commonest being *Pseudomanas aeruginosa* and *Acinetobacter baumanni*. There is an increasing trend of resistance in most isolates from ICU. In a recent study on VAP by Chaudhury *et al*., carbapenem resistance in non-fermenters was found to be as high as 33% (*4*). In a study done by Sharan *et al*., meropenem resistance in non-fermenters isolated from hospital acquired infections was found to be as high as 55% (*5*). The resistance in this study was higher compared to other Indian studies as

Table 2. Proportion of the resistant isolates for the commonest organisms

Antibiotic name	Aba	95% CI	Kp	95% CI	Pae	95% CI
Piperacillin/Tazobactam	79.7	68.4 - 87.8	74	59.4 - 84.9	52	42.9 - 61.0
Amikacin	91.9	82.6 - 96.7	82	68.1 - 91.0	40.8	32.2 - 50.0
Meropenem + Amikacin	86.5	76.1 - 93.0	70	55.2 - 81.7	28	20.5 - 36.9
Amoxicillin/Clavulanic acid	97.3	89.7 - 99.5	94	82.5 - 98.4	100	96.3 - 100
Cefotaxime	94.6	86.0 - 98.3	96	85.1 - 99.3	100	96.3 - 100
Ceftazidime	91.9	82.6 - 96.7	86	72.6 - 93.7	50.4	41.4 - 59.4
Cefoperazone/Sulbactam	50	38.3 - 61.7	74	59.4 - 84.9	54.4	45.3 - 63.3
Ciprofloxacin	91.9	82.6 - 96.7	86	72.6 - 93.7	57.6	48.4 - 66.3
Meropenem	89.2	79.3 - 94.9	74	59.4 - 84.9	48.8	39.8 - 57.9
Imipenem	79.7	68.4 - 87.8	56	41.3 - 69.7	53.6	44.5 - 62.5
Piperacillin/Tazobactam + Amikacin	75.7	64.1 - 84.6	70	55.2 - 81.7	29.6	21.9 - 38.5
Piperacillin/Tazobactam + Ciprofloxacin	77	65.5 - 85.7	74	59.4 - 84.9	45.6	36.7 - 54.7
Cefoperazone/Sulbactam + Amikacin	47.3	35.7 - 59.2	70	55.2 - 81.7	31.2	23.4 - 40.2
Cefoperazone/Sulbactam + Ciprofloxacin	45.9	34.4 - 57.8	74	59.4 - 84.9	46.4	37.5 - 55.5
Imepenem + Ciprofloxacin	75.7	64.1 - 84.6	54	39.5 - 67.9	34.4	26.3 - 43.5
Imipenem + Amikacin	77	65.5 - 85.7	50	35.7 - 64.3	28.8	21.2 - 37.7
Meropenem + Ciprofloxacin	87.8	77.6 - 93.9	74	59.4 - 84.9	44.8	36.0 - 53.9

Aba, *Acinetobacter baumanni.* Kp, *Klebsiella pneumoniae,* Pae, *Pseudomonas aeruginosa*.

the study was conducted in an apex referral centre which receives critically ill with history of receipt of multiple antibiotics. The resistance to carbapenems in our study was as high as 60%. The single antibiotic with the best activity against the organisms implicated for nosocomial pneumonia was cefoperazone-sulbactam. But the meagre 43% sensitivity of CS is no match to the high standards of 95% set by the clinical practice guidelines. The sensitivity of combination of antibiotics from two different classes (betalactam plus FQ/AG) was also a mere 56%. Therefore, adding a FQ or AG to betalactam gave an advantage of only 13% in our study. It has been noticed in previous studies, that indiscriminate use of FQs or AGs for other indications in tuberculosis endemic areas have led to high prevalence of FQ/AG resistant tuberculosis (*9*). In a country like India, where there is widespread fluoroquinolone and aminoglycoside resistance in tuberculosis, a risk-benefit analysis should be done before empirically prescribing FQ or AG as a part of combination therapy. In a study reported from Mumbai, fluoroquinolone and aminoglycoside resistance in tuberculosis increased from 39% and 4% respectively pre-2010 to 94% and 19% respectively post 2010 (*10*).

In a recent study, it was found that early effective empirical antibiotic therapy was associated with better outcomes in patients with carbapenem resistant *Acinetobacter baumanni* pneumonia (*11*). In a setting with high prevalence of resistance to betalactams, choosing an empiric regimen that will prove effective becomes difficult. Polymyxin resistance is reported from our country, mostly in form of case reports (*12*). The available literature suggests polymyxins as the most effective drug in these isolates. Colistin sensitivity is not done routinely in our setting as it requires micro-broth dilution testing for sensitivity reporting (*13*). Guidelines suggest avoiding polymyxins if alternative agents with adequate gram negative activity are available. With increasing resistance to betalactams, the clinicians in our setting are left with a difficult decision to ensure early appropriate antibiotic coverage and avoid superfluous treatment at the same time.

4. Conclusion

The commonly implicated organisms like *Pseudomonas* spp. and *Acinetobacter* spp. have high resistance to beta-lactam/beta-lactam inhibitor combinations and carbapenems. The sensitivity of empiric antibiotics is low in our settings. Urgent measures like Antimicrobial stewardship programmes and development of new drugs are needed to address the issue and save the polymyxins from becoming an empiric drug of choice.

Limitation

This was only a review of retrospective isolates including possible colonisers and contaminants. Further studies with clinically significant isolates are required to understand the complexity of the problem.

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Case Report

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Successful treatment of primary immune thrombocytopenia accompanied by diabetes mellitus treated using clarithromycin followed by prednisolone

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Summary **Macrolides have immunomodulatory effects including anti-inflammatory effects as well as antibacterial activity. In consideration of these immunomodulatory effects, we report a patient with primary immune thrombocytopenia (ITP) treated using clarithromycin (CAM), a macrolide, followed by prednisolone (PSL). A 78-year-old man with thrombocytopenia was admitted to our hospital for further examination. Initial laboratory data showed reduced platelet counts (1.7×10^4/μL). Finally, we diagnosed the patient as having primary ITP. Because the patient was suffering from diabetes mellitus (DM), he was treated with CAM as an alternative to PSL. The platelet count increased to 6.1×10^4/μL. The CAM treatment was terminated owing to gradual nausea and palpitation. During the CAM treatment, the DM was under control. We reinitiated treatment for ITP. The patient was successfully treated using PSL without severe hyperglycemia. This case shows that CAM treatment may represent a useful option for ITP patients who cannot receive PSL due to DM.**

Keywords: Immune thrombocytopenia, clarithromycin, prednisolone

1. Introduction

Macrolides such as clarithromycin (CAM) and erythromycin (EM), have not only antibacterial activity but also immunomodulatory effects including anti-inflammatory effects. In consideration of their immunomodulatory effects, we have previously reported several cases of immune thrombocytopenia (ITP) successfully treated using CAM or EM (*1-4*). We report herein a case of primary ITP accompanied by diabetes mellitus (DM) treated using CAM followed by prednisolone (PSL).

2. Case Report

A 78-year-old man with thrombocytopenia was admitted to our hospital for further examination. Physical examination revealed neither articular swelling nor skin rash suggestive of collagen diseases. His laboratory results included the following: white blood cell counts 5,480/μL (basophils 0.3%, eosinophils 4.2%, neutrophils 69.2%, lymphocytes 19.3%, monocytes 7.0%), hemoglobin (Hb) 10.0 g/dL, platelet count 1.7×10^4/μL, C-reactive protein 0.25 mg/dL, immunoglobulin (Ig) G 1,707 mg/dL, IgM 79 mg/dL, IgA 189 mg/dL, fasting blood sugar 150 mg/dL, and hemoglobin A_{1C} 6.5% (normal range, 4.6-6.2%). Neither antinuclear antibody nor rheumatoid factor was detected. The patient was negative for *Helicobacter pyroli* (HP) stool antigen using the enzyme-linked immunosorbent assay and for *HP* IgG antibodies. A bone marrow aspiration smear revealed normal bone marrow with a nucleated cell count of 90,000/μL and a megakaryocyte count of 55/μL without dysplasia or hemophagocytosis. No abnormal findings suggestive of infection were found in the systemic survey, including the chest roentgenogram and urinalysis. Based on these findings, we diagnosed the patient as having primary ITP. The patient was suffering from DM; therefore, in consideration of its immunomodulatory effects, we initially prescribed CAM (800 mg/day) as an alternative to PSL, after

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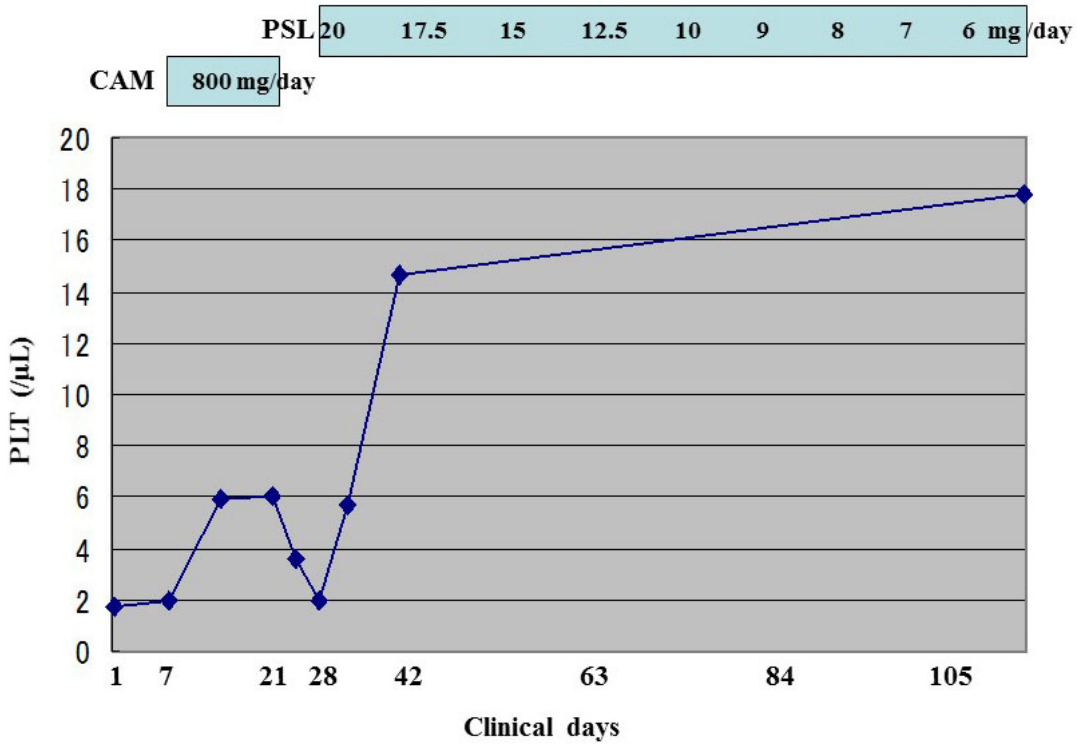

Figure 1. Laboratory data and prescribed agents on clinical days. CAM: clarithromycin, PSL: prednisolone, PLT: platelet

obtaining his informed consent. The clinical course is shown in Figure 1. Two weeks after CAM treatment, the platelet count increased from 2.0 to 6.1 × 10^4/μL. The CAM treatment was terminated owing to gradual nausea and palpitation that are probably adverse reactions of CAM. As a result, the platelet count decreased to 2.0 × 10^4/μL. During the CAM treatment, the DM was under control. We reinitiated treatment for ITP. This time the patient was administered PSL (20 mg/day). After 2 weeks, the platelet count increased to 14.7 × 10^4/μL, and he tolerated a gradual PSL tapering. By the end of the observation period, the platelet count increased to 17.8 × 10^4/μL on PSL (6 mg/day). During the treatment, the blood sugar was almost controlled and the patient presented no severe hyperglycemia episodes.

3. Discussion

Primary ITP is an acquired immune disorder characterized by an isolated thrombocytopenia due to pathogenic anti-platelet autoantibodies, T cell-mediated platelet destruction, and impaired megakaryocyte function. On the contrary, secondary ITP is triggered by inherited or acquired predisposing diseases such as chronic infections, including *HP* and human immunodeficiency virus, or autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis (*5*). Recent studies have suggested that *HP*-positive ITP patients can be successfully treated by eradication of the pathogen (proton pump inhibitor, amoxicillin, and CAM) (*6,7*). On the contrary, in primary ITP, first-line treatments include corticosteroids. We have previously reported several cases of primary and secondary ITP such as *HP*-positive ITP showing increased platelet counts following macrolides treatment (*1-4*). In those cases, we speculated that the ITP improved by the immunomodulatory effects of the macrolides or their anti-bacterial activity. In addition to the anti-bacterial activity, macrolides have immunomodulatory effects including anti-inflammatory activities and are used for diseases such as diffuse panbronchiolitis, organizing pneumonia, and rheumatoid arthritis (*8*). The macrolides have effects on neutrophil function (decreased oxidant production, apoptosis) and on the production of cytokines involved in the inflammation cascade (decreased production of IL-1, IL-6, IL-8, and TNF and increased production of IL-10 and, possibly, IL-4) (*9*). EM and its derivatives inhibit T lymphocyte proliferation and induce T lymphocyte apoptosis (*10*). EM has been shown to potentiate the function of regulatory T cells in a rat model (*11*). In the present case, considering our previous experience, we thought that CAM treatment would be effective for our primary ITP patient. Although the CAM had to be stopped due to nausea and palpitation, the DM was controlled during CAM treatment. Consequently, the patient was safely and successfully treated using PSL without severe hyperglycemia. Since older patients have a tendency to suffer from chronic diseases that are exacerbated by the use of corticosteroids, such as DM, osteoporosis, and hypertension, macrolides treatment may represent a

useful option for treating ITP in them. According to the present case, CAM treatment demonstrated the actual benefit to the ITP patient accompanied by DM.

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Case Report

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Prothrombin complex concentrate and fatal thrombotic adverse events: A complication to keep in mind

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Summary **Thromboembolic events such as deep vein thrombosis and pulmonary embolism are well-known complications that can occur after prothrombin complex concentrate therapy. However, acute myocardial infarction is a very rare but potentially life-threatening complication that was exclusively described in patients with bleeding disorders who received chronic and recurrent concentrate infusions. We report the case of a 70 year-old male patient with cholangiocarcinoma who was admitted to our hospital with worsening fatigue and weakness. His stay was complicated by uncontrolled bleeding secondary to rivaroxaban use and advanced liver disease. By the end of the prothrombin complex concentrate infusion used to reverse his coagulopathy, patient developed ST-segment elevation myocardial infarction with cardiogenic shock and passed away. This is the first reported case of acute myocardial infarction that occurs in a patient without hemophilia and after the first prothrombin complex concentrate infusion.**

Keywords: Prothrombin complex concentrate, thromboembolic adverse event, ST-segment elevation myocardial infarction, liver disease

1. Introduction

Oral anticoagulants are routinely prescribed for the prevention or treatment of thromboembolic events, with millions of prescriptions being issued for anticoagulation in the United States every year (*1*). On the other side, the risk of major hemorrhage increases dramatically with the use of anticoagulation reaching 1.7% to 3.4% in patients on vitamin K antagonists (VKA), for example (*2*). Prothrombin Complex Concentrate (PCC) has been approved for the reversal of VKA-associated major bleeding, but it is also often used off-label to reverse coagulopathy in patients receiving non-VKA anticoagulants or in patients with liver disease (*3,4*). Although PCC infusion can quickly reverse the anti-coagulation effect of VKA and save lives in certain critical situations, it is associated with increased risk of thromboembolic events such as pulmonary embolism, deep vein thrombosis, and cerebrovascular accident. Here, we present the first case of ST-segment elevation myocardial infarction (STEMI) secondary to PCC infusion in a patient with advanced liver disease.

2. Case Report

A 70 year-old man presented to our emergency department because of worsening generalized weakness over the last three days. The patient is previously known to have cholangiocarcinoma diagnosed six months ago when he developed diffuse jaundice and lower extremities edema. Back then, he was found to have multiple liver lesions with a CA 19-9 level of 10,616 u/mL. The liver biopsy demonstrated diffuse intra-hepatic cholangiocarcinoma, and he was started on regular cycles of chemotherapy with gemcitabine and oxaliplatin. At the same time, he was diagnosed with lower extremities deep vein thrombosis and was treated with rivaroxaban. Other medical history is relevant for hypothyroidism for which he was maintained

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on replacement therapy, and chronic kidney disease (Baseline estimated glomerular filtration rate around 57 mL/min). Among his home medications, he was also taking furosemide and spironolactone to control his ascites. He never smoked cigarettes, did not consume alcohol or used illicit drugs. At baseline, patient was fully oriented and ambulatory with a walker. At this time, he presented one week after his last chemotherapy cycle because of weakness and confusion. Family provided the history and said that for the last few days he was getting weaker, unable to ambulate on his own, and was getting confused from time to time. Upon evaluation, patient was afebrile, had a heart rate of 96 beats per minute, with a blood pressure of 83/57 mmHg. On physical examination, he was lethargic, confused, but arousable to painful stimuli. He was moving all his extremities and recognized his family members, but was not oriented to place or time. He was diffusely jaundiced and had bilateral lower field crackles on lungs examination. His abdomen was distended and dull to percussion, and he had bilateral lower extremities 3+ pitting edema. Blood work showed slightly decreased white blood cells count, with anemia (Hemoglobin of 10 g/dL) and thrombocytopenia (Platelets of 50,000/ uL). He also had acute kidney injury with a creatinine level of 2.3 mg/dL (Baseline of 1.6 mg/dL), and prolonged international normalized ratio (INR) and partial thromboplastin time (4.9 and 46.4 s respectively). Total bilirubin level was 6.3 mg/dL and aspartate and alanine aminotransferases were around two-times the normal level. Baseline electrocardiogram (ECG) showed right bundle branch block (Figure 1), and 2-D echocardiography was within normal range.

Patient was admitted to the intensive care unit for a presumed diagnosis of sepsis. He received broad spectrum antibiotics, resuscitated with fluids and maintained on low dose norepinephrine keeping his mean arterial blood pressure around 65 mmHg. Pancultures were drawn, Rivaroxaban was held, and abdominal paracentesis was performed urgently ruling out spontaneous bacterial peritonitis. Few hours later, patient started bleeding around his intravenous lines and from the site of paracentesis, and developed epistaxis. Emergent peripheral blood smear showed only low platelets count and macrocytic anemia without any schistocytes ruling out disseminated intravascular coagulation. The patient's coagulopathic state was attributed to both the use of rivaroxaban and worsening liver failure with prolonged INR secondary to the progression of his cholangiocarcinoma. Since rivaroxaban has no antidote commercially available on the market (*Andexanet Alfa* being still studied), the decision was taken to administer prothrombin complex concentrate (PCC) to try to control the bleeding. For a pre-treatment INR of 4.9, we administered PCC at a dose of 35 u/kg and at a rate of 0.12 mL/kg/minute. By the end of the infusion, the medical team noted some changes on the heart monitor. An ECG performed at that time showed evolving antero-septo-lateral ST-segment elevation myocardial infarction (Figure 2). Troponin level rose acutely from 0.02 to 3.95 ug/L. No cardiovascular intervention was possible at that time in the settings of anemia, thrombocytopenia, prolonged INR and active bleeding. Shortly after, patient went into cardiac arrest. All the efforts to resuscitate him were unsuccessful, and the patient expired.

3. Discussion

Adverse events following PCC infusion are common and can range from minor incidents such as headache, nausea or vomiting, to more severe and lethal

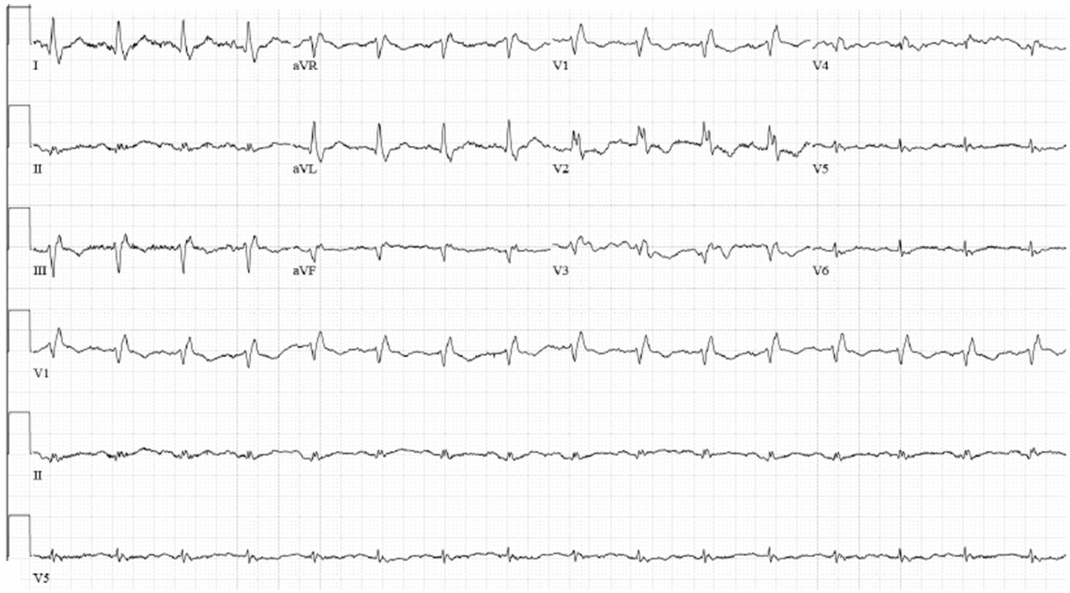

Figure 1. Baseline ECG. Initial ECG showing sinus rhythm with right bundle branch block.

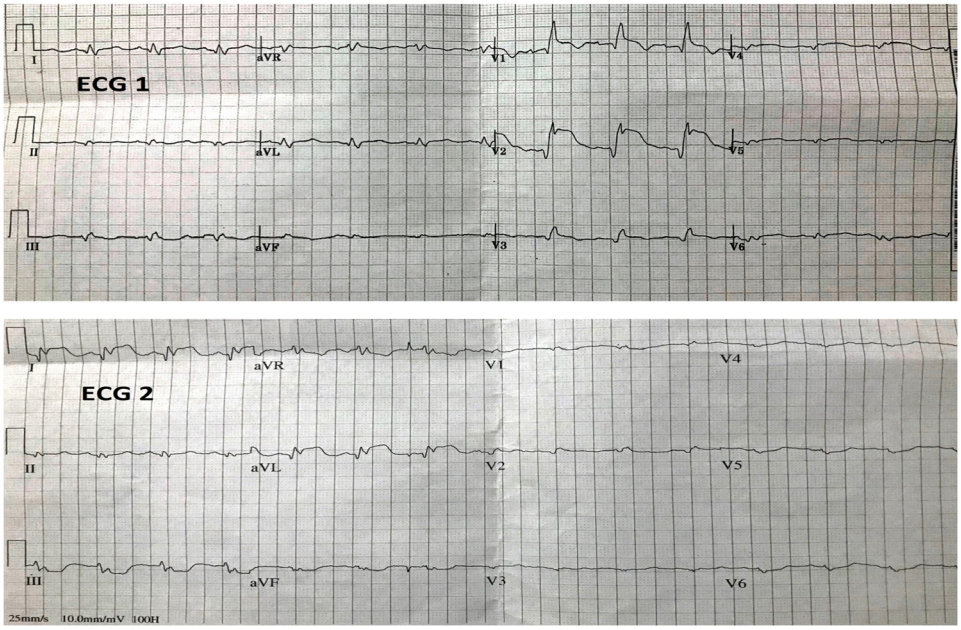

Figure 2. ECGs post-PCC infusion. Serial ECGs showing evolving antero-septo-lateral ST-segment elevation myocardial infarction after infusion of PCC.

complications suchlike a fatal thromboembolic event. Venous thromboembolism including deep vein thrombosis and pulmonary embolism are more common than arterial events such as cerebrovascular accident (*5*). Myocardial infarctions are actually quite unusual, but have been reported in few patients who suffered from hemophilia, after receiving large cumulative doses of PCC infusions (*6*). Our article describes the first case of STEMI that occurred in a PCC-naïve patient with no genetic bleeding disorder.

Myocardial infarction associated with the use of PCC is a well-described, but rare, clinical event. There have been around 16 such cases reported in the literature exclusively in patients with hemophilia who were exposed to recurrent infusions of PCC (*6*). Although some factors are known to increase the risk of venous thromboembolism complications of PCC therapy such as liver diseases and crush injuries, they do not appear to play a major role in the pathogenesis of myocardial infarctions related to the use of these concentrates (*6*). The management of such complication should it happens is debatable mainly because its pathogenesis remains poorly understood. Most previously reported cases were managed conservatively with opiate analgesia, nitrates, diuretics, anti-arrhythmics and inotropes (*6*). Anti-coagulants, anti-platelets, thrombolytic therapy or even percutaneous interventions are not a plausible or wise choice owing the high risk of bleeding complications in this population. Matter of fact, most of these patients who underwent post-mortem autopsy were surprisingly found to have myocardial hemorrhage without any evidence of coronary atherosclerosis or thrombosis (*6*). This might actually refute the assumption that myocardial infarction was caused by arterial thrombosis due to a hypercoagulable state produced by the infusion of PCC.

In the literature, no consensus is currently available for the treatment of PCC-induced myocardial infarction. We would suggest immediate stopping of the infusion, stabilizing the patient with symptoms control (analgesia/ nitrate) and hemodynamic support if needed. In any case, our patient was too sick to even consider any kind of revascularization approach or anti-thrombotic therapy in view of his active bleeding.

Keep in mind that prevention of complications is way better than treating them. For the time being and until new randomized controlled trials come up with a definitive answer, we advise against the routine use of PCC for coagulopathy reversal in patients with liver disease. However, PCC might be an option for prudently selected patients with excessive, life-threatening bleeding, after weighing risks and benefits of such therapy.

In conclusion, PCC is not a completely benign therapy and subsequent fatal complications can occur. Awareness of these adverse events and familiarity with the predisposing factors is crucial for avoiding and treating such complications should they arise. Finally, decision on PCC usage must be tailored on a case by case manner.

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Case Report

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Shewanella algae in a chronic suppurative otitis media patient with cholesteatoma

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Summary **We present *Shewanella algea* infection in a chronic suppurative otitis media (CSOM) patient with cholesteatoma in terms of clinical course and treatment. This is the first time *S. algea* is found as solely pathogen in a CSOM patient without history of contact with seawater in Turkey. The patient admitted to the hospital several times with complaints of otorrhoea, was diagnosed as otitis media and treated. He was hospitalized to the otorhinolaryngology department for further evaluation of recurrent infections. The patient was diagnosed as cholesteatoma according to computed tomography scan findings and was operated for cholesteatoma. As a result of surgical and medical treatment he was discharged with full recovery. Physicians must be aware of rarely seen pathogens and their unexpected ways of transmission and underlying causes such as cholesteatoma when treating patients for CSOM.**

Keywords: Shewanella, chronic suppurative otitis media, cholesteatoma

1. Introduction

The genus *Shewanella* are widely distributed in the environment as motile, non-fermentative, facultative anaerobe, saprophytic Gram-negative bacilli. *Shewanella* spp. is a member of the marine microflora and is a rare pathogen for human being. The prevalence of Shewanella infection is high in geographic regions with temperate climates such as parts of United States, South Africa, Australia, Asia and Southern Europe (*1,2*). *Shewanella* infections are also seen in Turkey due to climate features. Direct contact to seawater or consumption of contaminated seafood are well-known risk factors for infections (*3*). We present *Shewanella algae* in a chronic suppurative otitis media (CSOM) patient with cholesteatoma without exposure to marine environment.

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2. Case Report

A 34 year-old male was admitted to otorhinolaryngology clinic on December 17, 2016 with otorrhea and hearing loss. The patient had complaints of intermittent ear discharge for a long time and the hearing loss occurred afterwards. He was treated twice in the outpatient clinic in 2015. In December 2016 he was admitted to the hospital with the same complaints. According to physical examination findings, the patient was diagnosed as otitis media. Ofloxacin 0.3% ear drop and a corticosteroid ear drop was prescribed. The patient did not recover despite medical treatment. He was hospitalized in the otorhinolaryngology department for further evaluation on January 4, 2017. Emprical treatment with ampicillin-sulbactam 1 g 3 × 1 (IV), ofloxacin 0.3% ear drop 1 g 3 × 1, corticosteroid 1 mg ear drop were started. Ciprofloxacin (IV) 200 mg 3 × 1 was added to the treatment of the patient who did not give response to treatment. The patient did not report any contact with seawater or marine environment. The biochemical test result of C-reactive protein was 3.3 mg/L (reference 0-3.5 mg/L). Complete blood count and erythrocyte sedimentation rate results were within normal limits.

Samples of middle-ear discharge were collected

from the patient, by an otorhinolaryngology specialist, under strict aseptic conditions using sterile swabs, after cleaning the external auditory canal with a different swab. The swab samples were immediately sent to the microbiology laboratory for bacterial studies. The swab sample was cultured on 5% sheep blood agar and eosin methylene blue agar for isolation of aerobic bacteria, and incubated aerobically at 37°C for 24-48 hours. The isolates grown were analyzed according to standard microbiological and biochemical methods. After 24 hours of incubation non-fermentative colorless colonies were observed on eosin methylene blue agar while hemolytic mucoid colonies were noted on sheep blood agar. According to biochemical tests these organisms were catalase, oxidase positive and H_2S production was positive. Urea was hydrolysed. The solely isolated organism from middle-ear discharge was identified as *Shewanella algae* by Vitek2 Compact (bioMerieux, France) automated identification system.

According to antimicrobial susceptibility results which were obtained from VITEK 2 Compact system; the strain was sensitive to ceftazidime, imipenem, meropenem, amikacin, gentamycin, and ciprofloxacin and resistant to trimethoprim/sulfamethoxazole, cefuroxime, ampicillin/sulbactam and amoxicillin/clavulanic acid.

In microscopic ear examination, purulent discharge, epithelium and cholesteatoma were seen. In the computed tomography mastoid cavity was also filled with soft tissue consistent with cholesteatoma. Due to the antibiotic susceptibility results, treatment was continued with ciprofloxacin (IV) 400 mg. As soon as ear discharge ended, patient with CSOM was operated for cholesteatoma on January 11, 2017. The patient was discharged with full recovery as a result of medical and surgical treatment. The written informed consent was obtained from the patient for this study.

3. Discussion

CSOM is formed by chronic inflammation of the middle ear and mastoid mucosa in which the tympanic membrane intactness (perforation or tympanostomy tube) was disturbed and otorrhea is usually present. However there is no consensus about the duration of the symptoms. CSOM in some studies is described as otorrhea through a perforated tympanic membrane continuing for at least 2 weeks, whereas other studies accept this period lasting for 2-6 weeks. It is thought that CSOM develop after an unsuccessfully treated acute otitis media infection (*4*). In our case, the patient also suffered from CSOM.

Moreover recurrent CSOM is due to one or a combination of several factors. These include therapy with oral antibiotics alone, treatment with non-antibacterial drops, uncompliance with the treatment process, infection with resistant bacteria such as *P. aeruginosa* or MRSA, and/or the presence of a cholesteatoma. Disease can not be managed and tends to be recurrent in patients with a disrupted ear anatomy or who are prone to infections (*5*). He was admitted to the hospital two times with ear discharge complaints in 2015. According to physical examination findings, the patient was diagnosed as otitis media and treated empirically. In December 2016, he was applied to the hospital with the same complaints and did not respond to treatment. He was hospitalized for further analysis to investigate the underlying cause of recurrent infections.

Cholesteatoma is a well-demarcated non-cancerous cystic lesion derived from an abnormal growth of keratinizing squamous epithelium in the temporal bone. Cholesteatoma results from the enzymatic activity of the cholesteatoma matrix. This abnormal growth is locally invasive and capable of causing the destruction of structures in the middle ear cleft (*6-8*). The growth of cholesteatomas often progress undetected for years until they have become dangerously large and threaten to invade intratemporal structures and cause intra- and extracranial complications (*9,10*). Recurrent or persistent otorrhea over a period of 2 weeks should be included in differential diagnosis as a possible warning sign of cholesteatoma, particularly when these symptoms persist despite treatment or in cases involving a suspicious hearing loss in an ear that has previously been operated on (*11*). Our patient had recurring otorrhea several times persisting for more than 2 weeks.

It has been reported that *Shewanella* spp. are responsible for a wide range of clinical conditions such as skin and soft tissue infections, bacteremia, gastroenteritis, cerebellar abscesses, ear and eye infections, infective endocarditis and pericarditis. Direct contact to seawater or consumption of contaminated seafood are well-known risk factors for infections (*1,3,12,13*). Pneumonia, cerebellar abscess, meningitis and wound infections were the reported cases from our country (*14-16*). Yılmaz *et al*. reported a case of a cerebellar abscess and meningitis caused by *Shewanella putrefaciens* and *Klebsiella pneumoniae* in a fisherman, secondary to chronic otitis media (*15*). In a Danish study, the most common ear infection was due to *S. algea* and 47 out of 55 patients suffered from clinical symptoms shortly after seawater contact (*3*). Numerous studies in the literature have confirmed the relationship between marine contact and disease, but this relationship has not been shown in some studies (*12,17*). To *et al*. indicated that the seawater contact cannot be documented by some studies because of some limitations such as being retrospective which probably lack information on seawater contact (*12*). In our case we were able to obtain information that there was no seawater contact from the patient. Furthermore, the *Shewanella* infection rates increase during summer due to contact with marine environment (*12*). However the patient in our

study was admitted to otorhinolaryngology clinic first time in winter.

Shewanella isolates are usually found to be susceptible to third and fourth-generation cephalosporins, fluoroquinolones, aminoglycosides, erythromycin and chloramphenicol in many studies whereas they are less susceptible to trimethoprim/sulfamethoxazole. Some studies reported variable susceptibility results to ampicillin and cephalosporins (*2,3,18*). In accordance with these studies our *S. algea* isolate was susceptible to ceftazidime, imipenem, meropenem, amikacin, gentamycin, and ciprofloxacin whereas resistant to trimethoprim/sulfamethoxazole, cefuroxime, ampicillin/sulbactam and amoxicillin/clavulanic acid. In our case, it is the first time that *S. algae* was isolated as a sole pathogen in a patient with CSOM without a prior history of contact with seawater. In conclusion, physicians must be aware of these rare pathogens and underlying causes such as cholesteatoma when prescribing antibiotics for rarely seen pathogens in CSOM.

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Case Report

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Septic pulmonary emboli as a complication of peripheral venous cannula insertion

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Summary **Septic pulmonary emboli can occur as a complication of many diseases, most common being right sided infective endocarditis. Septic emboli through a peripheral venous cannula are rarely reported in literature though central venous catheter is commonly implicated. We describe a case of widespread cellulitis and septic pulmonary emboli as a complication of peripheral venous cannulation.**

Keywords: Cellulitis, septic emboli, cannulation

1. Introduction

Septic embolism can have varied presentations and clinical considerations. The major complication is due to vascular occlusion of the involved tissues or organs. Infected central venous catheters are commonly associated with septic emboli but peripheral vascular catheters are rarely implicated. We describe a rare case of septic pulmonary emboli related to infected peripheral venous cannulation caused by an unusual etiological agent.

2. Case Report

A 26-year-old gentleman of Uttarakhand, India, presented with complaints of fever, productive cough, sudden onset shortness of breath and cellulitis in both the upper limbs. He had been recently hospitalized for dengue fever and administered intravenous fluids through peripheral venous line. On physical examination, patient was febrile, tachypneic and in respiratory distress. On local examination, there were multiple pus filled bullae in left hand and swelling and erythema involving right forearm that had started at the site of cannulation. Chest examination showed active accessory muscles of respiration, stony dull percussion at the base of right lung and decreased breath sounds at right infrascapular, infraaxillary and mammary area. Other system examination was unremarkable. Chest X-ray revealed bilateral multiple patchy heterogeneous peripheral opacities and infiltrates with right sided pleural effusion. Contrast enhanced computed tomography (CECT) chest showed feeding vessel sign confirming the diagnosis as septic emboli (Figure 1). Venous Doppler and 2D-echocardiogarm were normal.

Laboratory findings showed marked leukocytosis (22,000/mm^3). Pus aspirate, blood sample and sputum sample were sent for microbiological testing. Patient was started empirically on ceftriaxone, vancomycin and clindamycin. The pus culture and sputum culture showed *Klebsiella pneumoniae* sensitive to cefoperazone-sulbactum, piperacillin-tazobactum, meropenem and amikacin. The antibiotics were modified accordingly to antimicrobial sensitivity profile to cefoperazone-sulbactum. Despite giving appropriate antibiotics, he continued to have fever though intensity of fever declined. Bronchoalveolar lavage (BAL) was done to confirm the etiology. The BAL sample was sent for galactomannan, fungal KOH mount and culture, AFB stain, geneXpert, gram stain, bacterial culture and sensitivity. BAL galactomannan, geneXpert and fungal culture were negative. BAL culture showed *Klebsiella pneumoniae* with same antimicrobial sensitivity profile. Diagnostic pleural tap was done. Laboratory findings of pleural fluid showed occasional lymphocytes in proteinaceous background. AFB stain, geneXpert and

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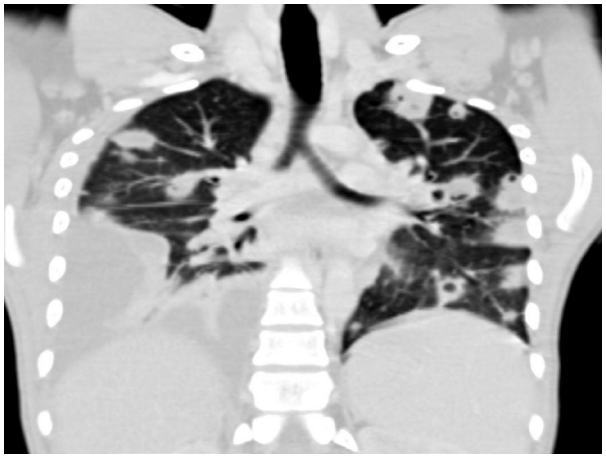

Figure 1. Contrast enhanced computed tomography chest image showing feeding vessel sign confirming the diagnosis as septic emboli

culture were sterile. On day 6 of starting cefoperazone-sulbactum, patient became afebrile. The skin lesions improved significantly. He was administered 2 weeks of cefoperazone-sulbactum and discharged on oral faropenem for 4 weeks. At the time of discharge, total leukocyte count (TLC) was 11,200/mm^3 with marked radiological resolution of lung opacities and healed skin lesions. He was followed up regularly. Chest X-ray and skin lesions showed complete resolution after 8 weeks.

3. Discussion

Catheter related blood stream infections are usually reported due to central venous catheters. Other complications seen due to venous catheters are thrombophlebitis, cellulitis, septic thrombophlebitis rarely tissue necrosis and septic emboli (*1*). Septic emboli refer to embolization of infectious particles in the circulation. Depending upon the site of embolization, the clinical presentation can vary.

There are various routes related to infection of peripheral venous catheter. These are extraluminal, intraluminal, hematogenous spread, and use of contaminated infusate (*2*). Extraluminal route is most common and infecting microorganisms are usually endogenous skin flora predominantly composed of gram positive bacteria. The skin organisms migrate through the narrow extraluminal space created due to insertion of intravenous catheter. Contamination of catheter hub can occur either due to skin organisms or inappropriate hand hygiene of healthcare worker. A study done by Lee WL *et al*. (*3*) found that continuous intravenous infusion more than 24 hours, use of infusion pumps, insertion of cannula in lower extremity and any pre-existing neurological and neurosurgical sequelae are independent risk factors that increase the chance of soft tissue infections related to peripheral intravenous cannulation.

The usual microorganisms in catheter related infections are gram positive bacteria accounting for almost two-third of cases. Among all, *Staphylococcus aureus* is the commonest. *Pseudomonas aeruginosa* is the most frequently grown gram negative bacteria from such infections, almost always associated with inappropriate health care practices (*4,5*). In the present case, the etiological agent was extended spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae*, an uncommon pathogen in such clinical presentation of cellulitis and septic pulmonary emboli. This drug resistant organism might have been acquired due to improper hand hygiene of health care worker.

Till date only couple of cases of septic pulmonary emboli through peripheral intravenous catheter have been reported in English literature. First case was reported by Friedberg *et al*. (*6*) from Australia. A patient of schizophrenia with altered sensorium had multiple septic emboli who succumbed to his illness. Another report is by Fidan *et al*. (*7*) from Turkey. The patient had septic embolus and recovered on intravenous antibiotics. In present case, the patient was an apparently healthy male, who developed this complication and recovered completely after 6 weeks of intravenous antibiotics.

This clinical entity is commonly seen in patients suffering from right sided bacterial endocarditis, infected central venous catheters, periodontal infection and prosthetic vascular devices. The most common symptom is fever (93%) followed by chest pain, respiratory distress, cough and hemoptysis (*8*). Respiratory symptoms are present in less than 50% of cases of septic pulmonary emboli and so demand a high index of clinical suspicion to promptly administer intravenous antibiotics for good prognosis.

This case highlights that a simple procedure of peripheral intravenous cannulation can lead to catastrophic complication of septic pulmonary emboli and widespread cellulitis if not done with proper care and precautions. Also the usual pathogens in such clinical settings are gram positive bacteria, but with the history of recent hospitalization empirical therapy should also cover drug resistant gram negative microorganisms. It also emphasizes the importance of appropriate healthcare practices to be taken care during all procedures.

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