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# Vitamin $K_2$ supplementation blocks the beneficial effects of IFN-α-2b administered on the early stages of liver cancer development in rats

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ABSTRACT

*Objective:* Vitamin $K_2$, which is present in dairy products and has been recommended as a micronutrient supplement in humans, contains anticancer properties. Interferon (IFN)-α-2b administered during development of hepatic preneoplasia decreased both number and volume percentage of altered hepatic foci (AHF) by increasing apoptosis in the foci. The aim of this study was to evaluate the effects of IFN-α-2b treatment supplemented with vitamin $K_2$ in the early stages of liver cancer development in rats.
*Methods:* Adult male Wistar rats were subjected to a two-phase model of hepatocarcinogenesis (initiated-promoted [IP] group). Animals were divided into four groups: untreated (IP), IP treated with IFN-α-2b ($6.5 \times 10^5$ U/kg), IP treated with vitamin $K_2$ (10 mg/kg), and IP treated with both compounds.
*Results:* The study results demonstrated that vitamin $K_2$ blocked IFN-α-2b–induced reduction in size and volume of the altered hepatic foci and inhibited IFN-α-2b–induced apoptosis. Its inhibition of IFN-α-2b–induced apoptosis was mediated by increased levels of total hepatic Bcl-2 in rat preneoplastic livers.
*Conclusion:* These findings demonstrate that supportive vitamin supplements or therapies are not always safe because they could put the life of patients treated with IFN-α-2b at risk.
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## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an estimated incidence of >1 million new cases annually [1]. Many cancer patients combine some forms of complementary and alternative medicine therapies with conventional treatment. The most common choice of these treatments is the nutritional administration of certain essential compounds such as vitamins [2,3]. Vitamin K is a fat-soluble essential vitamin. Its only known physiological role is as a cofactor for γ-glutamyl-carboxylase, which acts on several blood-clotting proteins [4]. Vitamin K is similarly involved in the regulation of bone metabolism by γ-carboxylation of bone matrix proteins [5].

There are three types of vitamin K: naturally occurring vitamin $K_1$ (phylloquinone), vitamin $K_2$ (menaquinone, MK), and chemically synthesized vitamin $K_3$ (menadione). Vitamins $K_1$ and $K_2$ are non-toxic even at high doses. Vitamin $K_3$ is toxic, causing hemolytic anemia, liver toxicity, and allergic reactions [6].

Vitamin $K_2$ is also known as MK-n (n = 1–14), where n stands for the number of repeating isoprenyl units in its side chain. The most common form of vitamin $K_2$ in animals is MK-4, which is produced by intestinal bacteria or is metabolically converted from other vitamin K types [7] (i.e., MK-7 shows a pharmacologic effect because it is converted to MK-4 in the liver) [8].

Vitamin $K_2$ is present in dairy products such as meat and eggs [9]. Epidemiologic studies have indicated that populations taking foods rich in micronutrients have lower incidence of cancer or cancer mortality [10]. In this connection, it was suggested that micronutrients may contain some anticancer properties [11]. Therefore, there is significant interest in determining whether vitamin $K_2$ offers protection against HCC [10,11].

There has been continuing interest in the use of vitamin $K_2$ in the chemoprevention of HCC in Asian countries because of its long-term safety [12]. It has been shown that vitamin K suppresses

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growth and induces apoptosis and differentiation in various cancer cells, including HCC cells in vitro and in vivo with little, if any, toxicity in adult HCC patients [13]. However, to our knowledge, the precise mechanism of its antitumoral action has not yet been clarified. A few studies have demonstrated reduction of HCC recurrence with vitamin $K_2$ supplementation; however, to confirm the beneficial effect or lack of it on vitamin $K_2$ administration, larger, better-quality trials are needed [14,15].

Recent studies have shown that vitamin $K_2$ can be combined with antitumor drugs to enhance their antiproliferative effects. It has been reported that the combined use of perindopril, an angiotensin-converting enzyme inhibitor drug, with vitamin $K_2$ attenuates preneoplastic hepatic lesions in rats [16]. Even more, a synergistic action that suppresses cell migration of human hepatoma cells has been demonstrated with sorafenib, the only drug approved by the FDA for the specific treatment of liver cancer [17].

IFN is known to be a multifunctional cytokine exhibiting various biological functions and has been used as an antiviral treatment in patients with chronic hepatitis C [18]. IFN-α has been shown to induce apoptosis in tumor cells in vivo using a similar dose to that used clinically [19]. It has been demonstrated that IFN-α applied in the early stages of tumor development could have an important clinical effect [20]. In this regard, we demonstrated that IFN-α-2b administered during the development of hepatic preneoplasia significantly decreased both number and volume of altered hepatic foci (AHF) by an increased programmed cell death in the foci. The apoptotic effect of IFN-α-2b on preneoplastic livers was mediated by an increase in reactive oxygen species and depends, to a large extent, on the deregulation of the Bcl-2 family, with a substantial increase in Bax levels in mitochondria and overexpression of p53 [21].

In the present study, we evaluated the effects of adding vitamin $K_2$ to the traditional IFN-α-2b treatment in the early stages of liver cancer development in rats. The effects of this combination on the development of liver cancer have not been tested experimentally in vivo.

## Materials and methods

### Chemicals

Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), and vitamin $K_2$ (MK-4) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). IFN-α-2b was obtained from BioSidus Laboratory (Buenos Aires, Argentina) and anti-P class of rat glutathione *S*-transferase (rGST-P) antibody from Stressgen Bioreagents (Ann Arbor, MI, USA). Antibodies against Bax, Bcl-2, Bcl-$x_L$, cytochrome c, proliferating cell nuclear antigen (PCNA), cyclin D1, p27, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pierce-enhanced chemiluminescence Western Blotting Substrate was from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were of the highest grade commercially available.

### Animals and treatment

Adult male Wistar rats (from ICIVET Litoral, UNL-CONICET; weight 250–280 g) were maintained in suspended stainless steel wire-bottom cages to prevent coprophagy (two per cage) in a room at constant temperature with a 12-h light/dark cycle, with food and water supplied ad libitum. Experimental protocols were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local animal care and use committee.

All animals were subjected to a two-phase model of rat hepatocarcinogenesis (initiation-promotion [IP] group). An overview of the experimental setup is provided in Figure 1A. The initiation stage was performed by the administration of two intraperitoneal necrogenic doses of DEN (150 mg/kg body weight [BW]) 2 wk apart. Administration of 2-AAF was performed 1 wk after the final injection of DEN. The 2-AAF was dissolved in dimethyl sulfoxide and then suspended in corn oil to a final concentration of 8 mg/mL. The rats received 20 mg/kg body weight of 2-AAF/corn oil suspension by gavage for 3 d/wk consecutively for 3 wk. After the IP treatment, animals were divided into four groups of five rats each: IP, IP-IFN (IFN group), IP-VK2 (VK2 group) and IP-IFN + VK2 (group IFN + VK2). Group IFN received IFN-α-2b ($6.5 \times 10^5$ U/kg BW) intraperitoneally three times per week for 3 wk. The dose of IFN-α-2b used was comparable to that used for therapeutic purpose [22]. Group VK2 received 10 mg/kg vitamin $K_2$ [23] by gavage, daily three times per day for 3 wk (vitamin $K_2$ was previously dissolved in corn oil). Group IFN + VK2 received both drugs at the doses administered individually.

At the end of week 9 of treatment, rats were bled through a cardiac puncture after administration of ketamine-xylazine (70 and 2.1 mg/kg BW, respectively), and livers were removed and processed.

### Immunohistochemical studies

#### Quantitation of AHF

rGST-P has been described as the most effective single marker of hepatic preneoplasia in the rat. Thus, immunohistochemical detection of rGST-P is the most widely used method for identification, quantitation, and assessment of rat AHF [24]. Liver slices from different lobes were fixed in 10% v/v formalin solution and embedded in low melting paraffin. Immunohistochemical staining was performed using the antibody raised against rGST-P. The slices were incubated with biotinylated goat antirabbit secondary antibody and then with horseradish peroxidase-conjugated streptavidin (HRP; CytoScan Detection Kit, Cell Marque, Rocklin, CA, USA). Signals were detected with DAB Substrate Kit (Cell Marque TM) https://www.cellmarque.com/cms/_downloads/errata/vol11/2016_Catalog_DIGITAL_Rev.0.2_p282.pdf followed by hematoxylin counterstaining. A representative number of field sections (usually between 1 and 1.5 $cm^2$ of tissue) was evaluated per animal. Images were collected using a CCD color video camera (Sony SSC-c370) attached to a Zeiss Axiolab microscope. Images were processed using a NIH imaging analysis system (ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The number of AHF per liver and AHF as percentage of liver were calculated according to the modified Saltykov's method [25] using the digitized images.

#### Determination of proliferative index

To investigate differences in proliferation activity in rGST-P–positive foci between the experimental groups, serially sectioned slides were examined by immunohistochemical staining with anti–rGST-P and anti-PCNA antibodies. PCNA was visualized by the method of Greenwell et al. [26] using as primary antibody anti-PCNA from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A representative number of random fields of liver sections (400 × magnification) were evaluated and scored. All PCNA-positive cells in G1, S, M, and G2 phases were combined as total proliferating cells. The number of proliferating cells within rGST-P–positive lesions was determined by examining ≥1000 hepatocytes. Proliferative index (PI) was expressed as proliferating cells scored per 1000 hepatocytes.

For Bcl-2 immunohistochemistry, serially sectioned slides were examined by immunohistochemical staining with anti–rGST-P and anti–Bcl-2 antibodies. Bcl-2 was visualized by the method of Greenwell et al. [22] using as primary antibody anti–Bcl-2 from Santa Cruz Biotechnology.

#### Hepatic collagen evaluation

Liver fibrosis was evaluated by digital image analysis on sections stained with 1% Direct Red 80/picric acid. This semiquantitative technique allows the estimation of total amount of hepatic collagen using several microphotographs slices obtained from each liver slice, as previously described [27]. Results were expressed as area occupied by collagen/area of the picture (in $cm^2$).

#### Immunoblotting

Tissue samples were homogenized in 150 mM KCl with protease inhibitors. Cytosolic, mitochondrial, and nuclear extracts were prepared as described previously [28,29]. The homogenates were centrifuged at 1000*g* to remove unbroken cells, nuclei, and heavy membranes. Nuclei were sedimented by centrifugation at 1000*g* (first centrifugation). Pellets from first centrifugation were washed and resuspended in radioimmunoprecipitation assay buffer containing 20 mmol phosphate-buffered solution (pH 8.0), 1% Triton, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mmol EDTA, 200 mmol sodium chloride, and protease inhibitors. Pellets were incubated on ice for 1 h and centrifuged (8000*g*, 15 min, 4°C) to obtain nuclear fraction. Supernatant from first centrifugation was used to obtain mitochondria fractions by centrifugation at 9000*g* at 4°C for 15 min. Supernatant from this centrifugation was then washed in $CaCl_2$ 0.1M and centrifuged at 27 000*g* and 4°C for 15 min. Pellet was discarded, and supernatant was kept as the cytosolic fraction.

Protein concentration was determined by the Lowry method [30]. Equal amounts of protein were subjected to electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride membranes (LD400 microplate reader, Beckman Coulter Inc, Fullerton, CA, USA). Membranes were blocked with T-phosphate-buffered saline 10% nonfat milk, washed and incubated overnight at 4°C with primary antibodies. Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by the Pierce-enhanced chemiluminescence detection system and quantified by densitometry using the Gel-Pro Analyzer software. Both equal loading

and protein transfer for each membrane were checked by incubations with the proper antibodies and by Ponceau S staining.

*Caspase-3 activity assay*

Caspase-3 activity was determined using EnzChek1 Caspase-3 Assay Kit #1 (Molecular Probes Inc., Eugene, OR, USA), according to the manufacturer's suggestions. Briefly, hepatic total homogenates from each sample were mixed with substrate solution. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a DTX 880 multimode detector (Beckman Coulter Inc, Fullerton, CA, USA).

*Statistical analysis*

Data are presented as means ± Standard Error of the Median (SEM). Statistical significance was evaluated by unpaired two-tailed Student's *t* test. Time-course studies were evaluated by two-way analysis of variance followed by Bonferoni post-test (GraphPad PRISM 4 software, GraphPad, San Diego, CA, USA). $P < 0.05$ was interpreted as a significant difference.

## Results

*General findings*

During the experiment, food consumption was similar in all groups and no signs of dehydration were observed along the treatment (data not shown). The ratio of liver to body weight was similar in all groups. Hematoxylin and eosin staining and further examination of the liver showed basically no differences between livers from all different groups (Fig. 1B) with no signs of acute or chronic inflammation despite AHF development (arrows in Fig. 1B). No significant differences were found in the activity of the hepatic enzymes aspartate transaminase and alanine aminotransferase in plasma (Fig. 1C).

*Vitamin $K_2$ blocks IFN-α-2b–induced reduction in size and volume of the AHF*

Changes in number and volume percentage of the liver rGST-P–positive foci are shown in Fig. 2. The number and volume percentages of AHF per liver in the IFN group were significantly decreased compared with the IP group (−39% and −65%, respectively). Surprisingly, VK2 did not show any differences on the number and volume percentages of AHF per liver with respect to the IP group, alone (VK2 group) or combined with IFN (IFN + VK2 group). Furthermore, the animals that received both drugs showed a trend toward an increment in number and volume percentages of AHF per liver, although this trend was not statistically significant.

*Vitamin $K_2$ does not improve total collagen deposition*

Collagen deposition upon the different treatments is shown in Fig. 3. As expected, IFN-α-2b treatment markedly reduced hepatic collagen deposition, therefore the degree of fibrosis compared with IP rats (−32 %). In line with results for the number and volume percentage of liver occupied by AHF, VK2 alone did not show any effects on collagen deposition. However, a reduction was observed for the combined IFN + VK2 group (−31%), similar to IFN group, indicating that the vitamin treatment did not have an additive action on the beneficial effects of IFN-α-2b.

*Vitamin $K_2$ partially blocks the apoptotic effect of IFN-α-2b*

As previously shown, immunoblot analysis of proapoptotic protein Bax showed an increase in mitochondrial protein expression in IFN as well as in the IFN + VK2 group (+219% and +97%, respectively) respective to IP. No changes were observed in the VK2 group (Fig. 4A). Levels of cytosolic cytochrome c were evaluated by immunoblotting. Cytosolic cytochrome c increased in IFN group compared with IP animals (+135%); the other groups did not show any differences respective to IP (Fig. 4B).

It was previously demonstrated that p53 induces apoptosis in HCC [31]. To determine whether p53 is a major mediator of apoptosis in preneoplastic foci of rats treated with IFN-α-2b, we performed immunoblot analysis of p53 using nuclear fractions. Administration of IFN-α-2b increased the expression of p53 (+75, 2%) protein compared with IP group, whereas vitamin $K_2$ treatment reduced the levels (−54, 3%) of the protein (Fig. 4C).

Also, the caspase cascade is now believed to be the main phatway by which the apoptosis is orchestrated. The most prevalent caspase in the cell is caspase-3 [32]. We determined caspase-3 activity in all studied groups. Figure 4D shows that caspase-3 activity was significantly increased in the IFN group compared with IP animals. Vitamin $K_2$ had no effects on caspase-3 activity in any of the other groups.

*Vitamin $K_2$ blocks the apoptotic effect of IFN-α-2b through an induction of the antiapoptotic protein Bcl-2*

Regarding the antiapoptotic protein Bcl-$x_L$, no significant differences were observed for any groups; however, a non-statistically significant trend to a decrease was observed in the IFN group compared with the IP group (Fig. 5A). The analysis of the antiapoptotic protein Bcl-2 showed an increase in mitochondrial protein expression in VK2 (+94%) and IFN + VK2 (+43%) groups (Fig. 5B). Interestingly, when Bcl-2 was evaluated by immunohistochemistry, a very interesting distribution pattern was found. An astonishing staining outside the AHF was observed in the IP group. The IFN group, however, presented with less stained cells, but Bcl-2–positive cells were distributed both inside and outside the AHF. The VK2 group presented with a pattern similar to the IP group; but in contrast, many Bcl-2–positive cells were found within the AHF. A similar pattern was observed in the combined group (Fig. 5C).

Finally, Bax-to-Bcl-2 ratios were calculated for each group. Arbitrarily setting the IP ratio as 1, we found a significant increase only in the IFN group ratio (3.2 ± 0.2), reinforcing our previous results where IFN-α-2b induced apoptosis in preneoplastic livers in rats (Fig. 5D).

*Vitamin $K_2$ does not affect cell proliferation and the cell cycle*

Figure 6A and Table 1 show the analysis of PCNA protein by both immunoblotting and immunohistochemistry, respectively. Immunoblotting showed no differences in the expression of PCNA protein between all the studied groups. In line, no changes were observed in the PI within the foci.

One proposed antitumoral mechanism of vitamin $K_2$ is its ability to arrest the cellular cycle [6], thus we measured the expression of two key proteins involved in the cycle: cyclin D1 and p27. Analysis of protein expression in nuclear extracts showed no differences in the expression of cyclin D1 protein in any of the studied groups (Fig. 6B). There was an increase in p27 protein in the IFN group compared with the IP group (+113%; Fig. 6C). However, its expression level is not sufficient to show any effects on cell cycle arrest.

## Discussion

Chemotherapy remains one of the main approaches for the treatment of HCC [33]. Despite significant advances in HCC management, there are no effective chemoprevention policies, and only sorafenib treatment has been approved for patients with advanced tumors [34]. However, despite its survival benefit (3 mo versus placebo patients) [35], sorafenib efficacy is now being debated because resistance and intolerance to it are very common. Thus, new and old systemic therapies need to be (re)considered for HCC patients with resistance or intolerance to sorafenib.

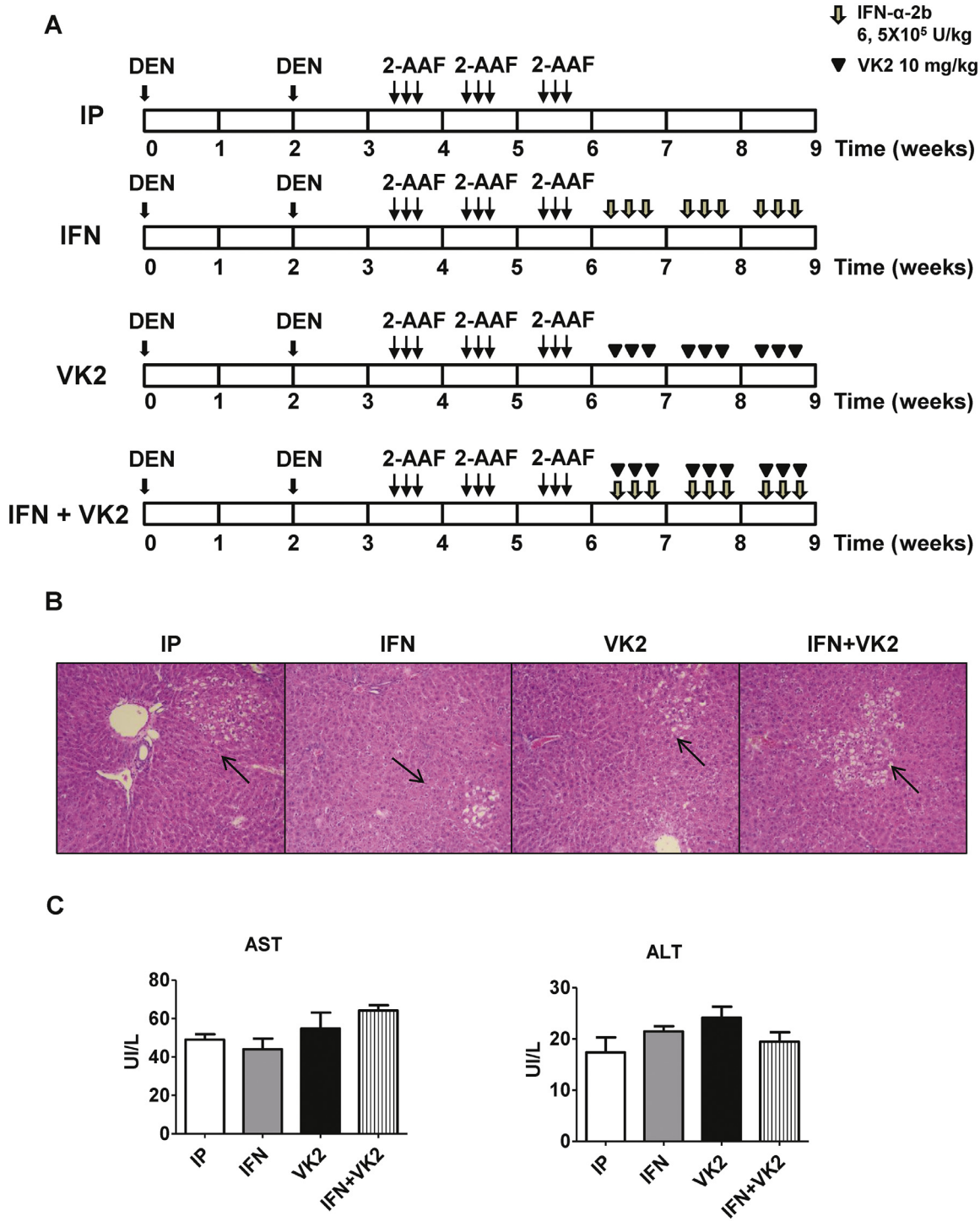


**Fig. 1.** No signs of liver damage in animals undergoing hepatic preneoplasia. (**A**) Animal treatment protocol. Male Wistar rats were subjected to a two-stage model of hepatocarcinogenesis. IP group received two intraperitoneal doses of DEN (150 mg/kg BW) 2 wk apart. One week after the last injection, the animals received 20 mg/kg BW of 2-AAF by gavage over 3 d/wk consecutively for 3 wk. Group IFN: IP rats received IFN-α-2b $6.5 \times 10^5$ U/kg intraperitoneally three times per week for 3 wk (weeks 6–9); group VK2: IP rats received 10 mg/kg vitamin $K_2$ orally three times per day for 3 wk (weeks 6–9); and group IFN + VK2: IP rats received both compounds at equal doses as per separate. The animals were sacrificed at the end of week 9. (**B**) Liver H&E staining for evaluation of hepatic architecture (100 ×). No signs of acute or chronic inflammation despite the AHF development (arrows). (**C**) Plasma transaminase levels, as a hepatic damage marker. 2-AAF, 2-acetylaminofluorene; AHF, altered hepatic foci; BW, body weight; DEN, diethylnitrosamine; H&E, hematoxylin and eosin; IFN, interferon; IP, initiated-promoted; PI, proliferative index; VK2, vitamin $K_2$.

The use of antivirals and vaccination has successfully diminished the incidence of hepatitis B–related HCC [34]. In this sense, IFN-α has been clinically used for delaying the progression of liver function impairment or for the prevention of HCC development in patients with chronic hepatitis B or C [36,37]. The clinical outcome of IFN-α monotherapy has not been satisfactory [38]; however, several studies have reported a strong antitumor activity and survival benefit of IFN-α-based combination therapy in HCC [39,40].

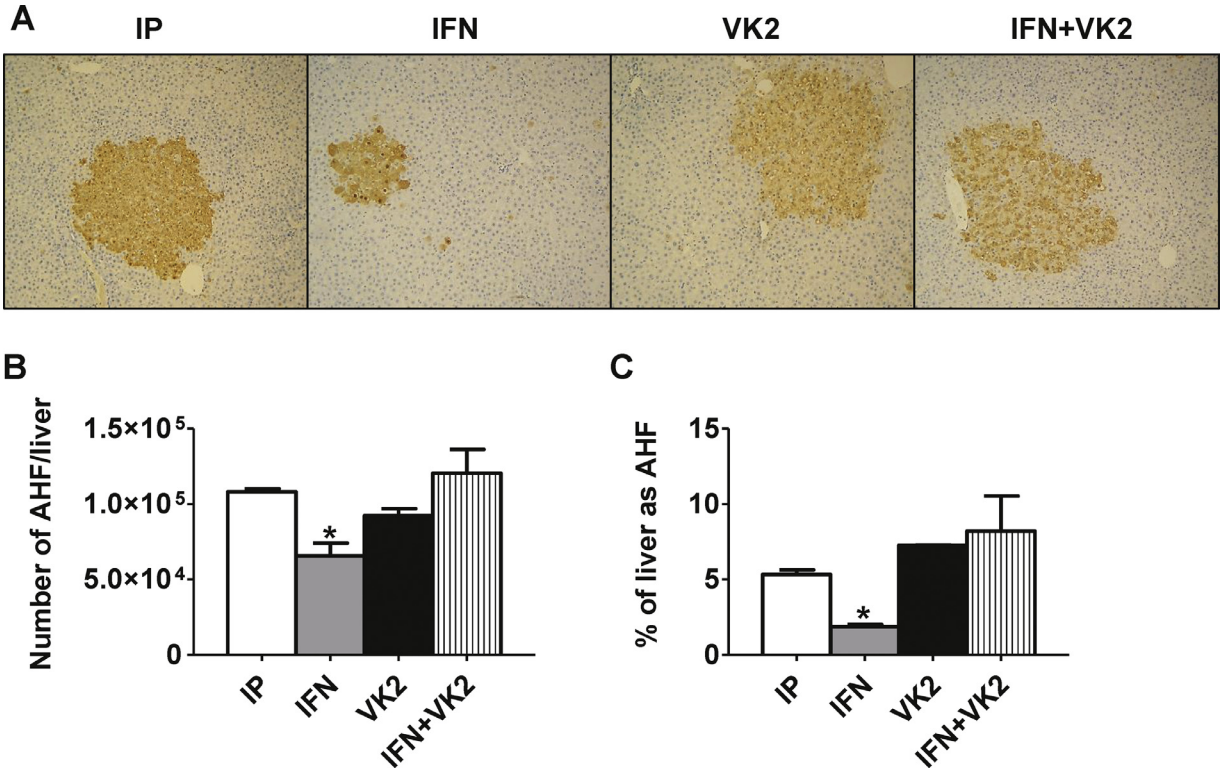


**Fig. 2.** Liver immunohistochemical study of rGST-p expression. (**A**) Representative images showing the size of rGST-p–positive foci in IP, IFN, VK2, and IFN + VK2 groups, magnification 100 × . Changes in (**B**) number of AHF per liver and (**C**) percentage of liver as AHF. Each bar indicates mean ± SEM. *$P < 0.05$ versus IP group. AHF, altered hepatic foci; IFN, interferon; IP, initiated-promoted; PI, proliferative index; rGST-P, anti-pi class of rat glutathione *S*-transferase antibody; VK2, vitamin $K_2$.

Also, patients undergoing IFN-α-2b treatment are susceptible to several secondary effects [37]. Thus, combining certain alternative medicinal therapies, such as vitamins, with conventional therapies is a common choice for many cancer patients. It is believed that these complementary therapies strengthen the general weakness experienced by these patients.

Several clinical trials have attempted to elucidate the role of vitamin $K_2$ in preventing the development of HCC in women with viral cirrhosis and in the suppression of recurrence of HCC, leading to greater survival [41,42]; however, results are still controversial [42–44].

Our group has been devoted to the study of the early stages of liver carcinogenesis for many years. In this sense, we found that administration of IFN-α-2b during the development of hepatic preneoplasia prevents the increment in the number and size of AHF [21,45]. In this study, we sought to evaluate the effect of

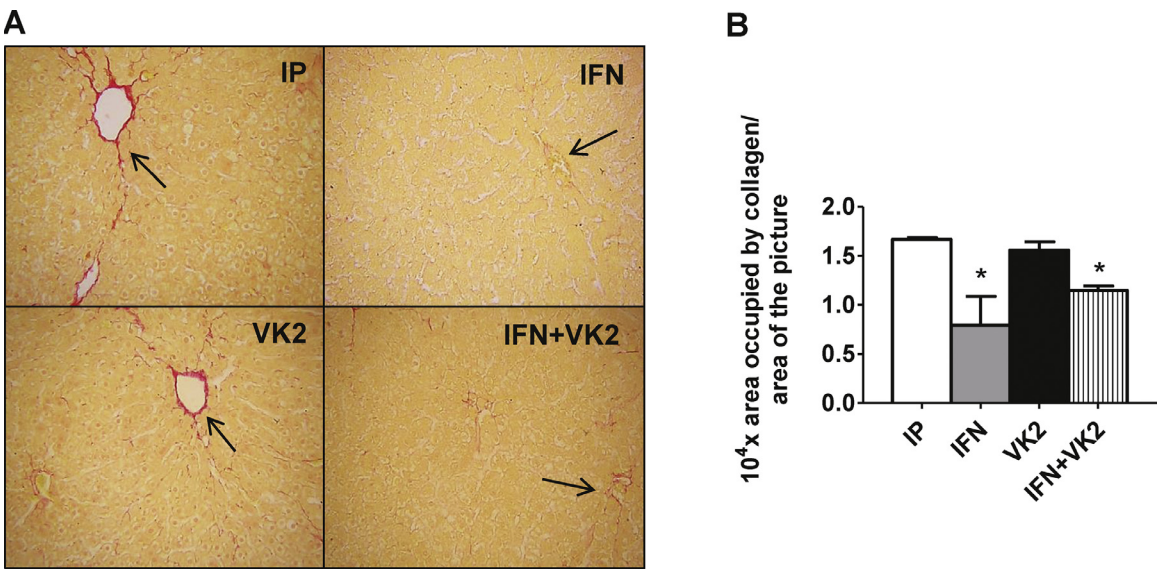


**Fig. 3.** Study of collagen deposition in the liver. (**A**) Representative images of liver collagen deposition. Collagen fibers are showed in red color (arrows), magnification 200 × . (**B**) Total liver collagen estimation. Each bar represents the mean ± SEM. *$P < 0.05$ vs IP group. IP, initiated-promoted.

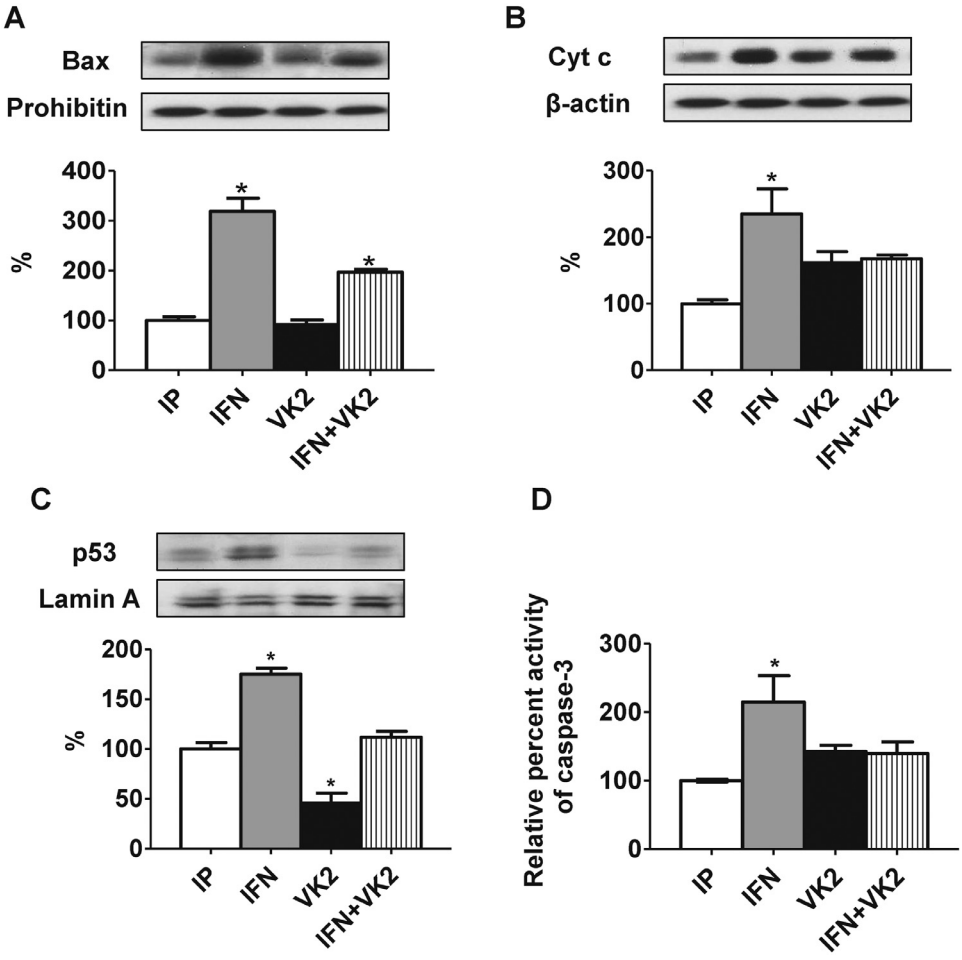


**Fig. 4.** Analysis of hepatic proapototic markers. (**A**) Study of Bax mitochondrial levels, (**B**) cytochrome c cytosolic levels, and (**C**) p53 nuclear levels by immunoblotting. The bars below each immunoblot panel represent the densitometric analysis of the bands. Each bar represents the mean ± SEM. (**D**) Caspase-3-like activity in hepatic total homogenates. Data are expressed in percent values as mean ± SEM; caspase-3 activity in IP group is considered 100%. *$P < 0.05$ vs IP group. IP, initiated-promoted.

IFN-α-2b administered after the end of the carcinogenic treatment, in order to compare the reversion effect of IFN-α-2b rather than the prevention effect. To our knowledge, this is the first study showing the revertive effects of IFN-α-2b in a rat model of early hepatocarcinogenesis. In addition, we also evaluated the revertive effect of vitamin $K_2$ using the same model and studied whether the combination of both compounds had a beneficial (additive or synergic) effect on liver cancer development compared with the individual treatments. In these sense, we found interesting but unexpected results as combination of IFN-α-2b and vitamin $K_2$, at previously individually tested doses [21,23], did not show any beneficial effects on the development of liver cancer. Furthermore, vitamin $K_2$ appears to have a blocking effect, as presented in the current report and in all the previously reported IFN-α-2b actions [21,28,45].

Here, we reported that vitamin $K_2$ blocks IFN-α-2b–induced reduction in size and volume of the AHF. A very thorough study of the vitamin $K_2$ dose to be administered was made before the start of the treatments. Doses ranging between 3 and 20 mg/kg were found in literature. Based on the AHF-reducing effect of vitamin $K_2$, we used the dose of 10 mg/kg BW administered 3 d a week for 3 wk. Yoshiji et al. reported an AHF-reducing effect using 3 mg/kg after a partial hepatectomy over an 8-wk period [16]. Despite using 10 mg/kg, vitamin $K_2$ was not able to reduce the number or the volume of the AHF. However, it is interesting to note that vitamin $K_2$ combined with IFN-α-2b had absolutely no effect on the development of the AHF; that is, AHF sizes and numbers were no different from the foci from IP livers, showing that vitamin $K_2$ would signal through a pathway interfering with the IFN-α-2b signaling pathway.

IFN-α is an interesting agent against liver fibrosis. Intrinsic antifibrogenic properties of this compound has been established by in vitro studies [46,47]. Indeed, IFN-α exerts its antifibrogenic effects in different rat models of hepatic fibrosis [48,49] and also in patients with hepatitis C virus [50]. We previously found that IP rats presented exacerbated liver fibrosis compared with control rats [51]. In this study, we showed that IFN-α-2b treatment improved the deposition of total collagen in IP rats. Interestingly, vitamin $K_2$ had no effect on collagen deposition, neither alone nor with IFN-α-2b. In this regard, to our knowledge, a beneficial effect of vitamin $K_2$ has never been reported on collagen deposition. It has been claimed that vitamin $K_2$ may contribute to collagen assembly and deposition in osteoblastic cells [52,53].

Vitamin $K_2$ induces apoptosis in a wide range of tumoral cells [7,54–58]. Unexpectedly, we did not find any signs of apoptosis induced by vitamin $K_2$ in our model. As previously shown in a model of prevention of hepatocarcinogenesis [21], we found that IFN-α-2b induces Bax translocation to the mitochondria in livers

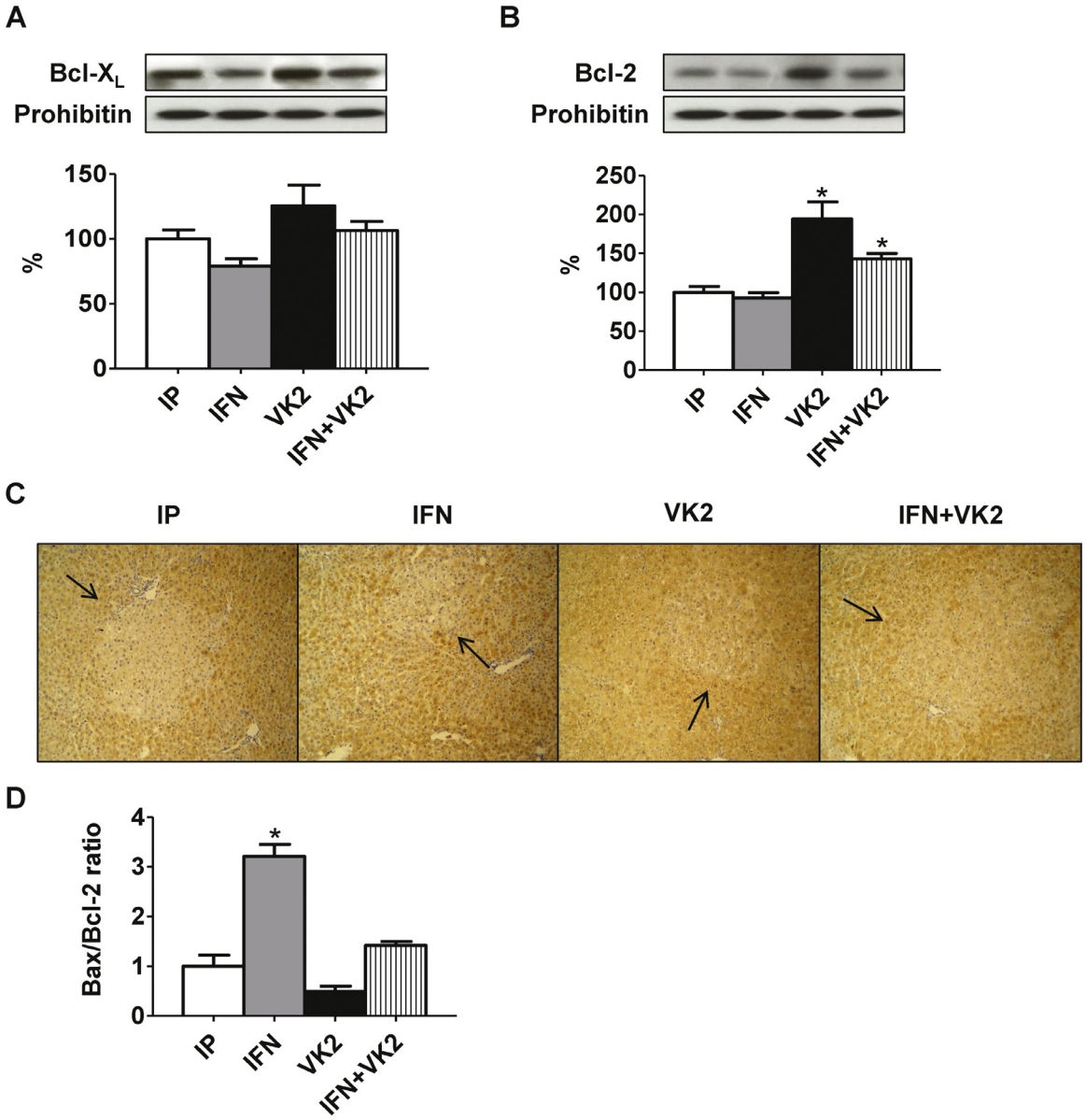


**Fig. 5.** Analysis of hepatic antiapoptotic markers. (**A**) Expression levels of Bcl-$X_L$, and (**B**) Bcl-2 in liver mitochondrial fractions by immunoblotting. The bars below each immunoblot panel represent the densitometric analysis of the bands. (**C**) Immunostaining for Bcl-2. Immunohistochemistry was performed using paraffin-embedded liver sections. The arrows show the presence of the AHF (100 ×). (**D**) Bar graph showing the ratio between optical density for Bax bands over Bcl-2 bands for individual immunoblottings (apoptotic index): Bax/Bcl-2. Results are expressed relative to IP group, to be considered 1. Bars represent mean ± SEM. $^*P < 0.05$ vs IP group. AHF, altered hepatic foci; IP, initiated-promoted.

from IP rats, inducing apoptosis; however, in this model the combination with vitamin $K_2$ seemed to block the FN-α-2b apoptotic effect. A similar pattern was found when other apoptotic proteins and activities were studied. IFN-α-2b–induced apoptosis is blocked by vitamin $K_2$, with no apoptotic effect of the vitamin alone. In this sense, it was previously reported that vitamin $K_2$ did not show apoptosis on Huh7 cells, where the effect appears only when vitamin $K_2$ is combined with another compound [59]. When p53 protein expression was evaluated, vitamin $K_2$ alone induced a marked decrease in protein expression.

Finally, we found that the levels of antiapoptotic protein Bcl-2 were increased when IP rats were treated with vitamin $K_2$ alone. This increment impairs the mithocondrial apoptotic pore formation, leading to a decreased or null apoptosis activation [60]. Furthermore, when the hepatic apoptotic index was calculated, no global effects of vitamin $K_2$ on apoptosis were found, showing once again that vitamin $K_2$ has a blocking effect on the IFN-α-2b–induced apoptotic effect.

It is interesting to note that Bcl-2 acts on inhibiting vitamin $K_2$ apoptotic action. Furthermore, it was demonstrated that the antitumor effect of vitamin $K_2$ might be improved by silencing BCL-2 expression in HCC [61].

In addition, programmed cell death is facilitated in part through the production of free radicals via oxidative pathways. In this connection, it has been proposed that Bcl-2 acts inhibiting cell death by interfering with the production of oxygen-derived free radicals induced by a wide variety of stimuli such as IFN-α-2b [45,62].

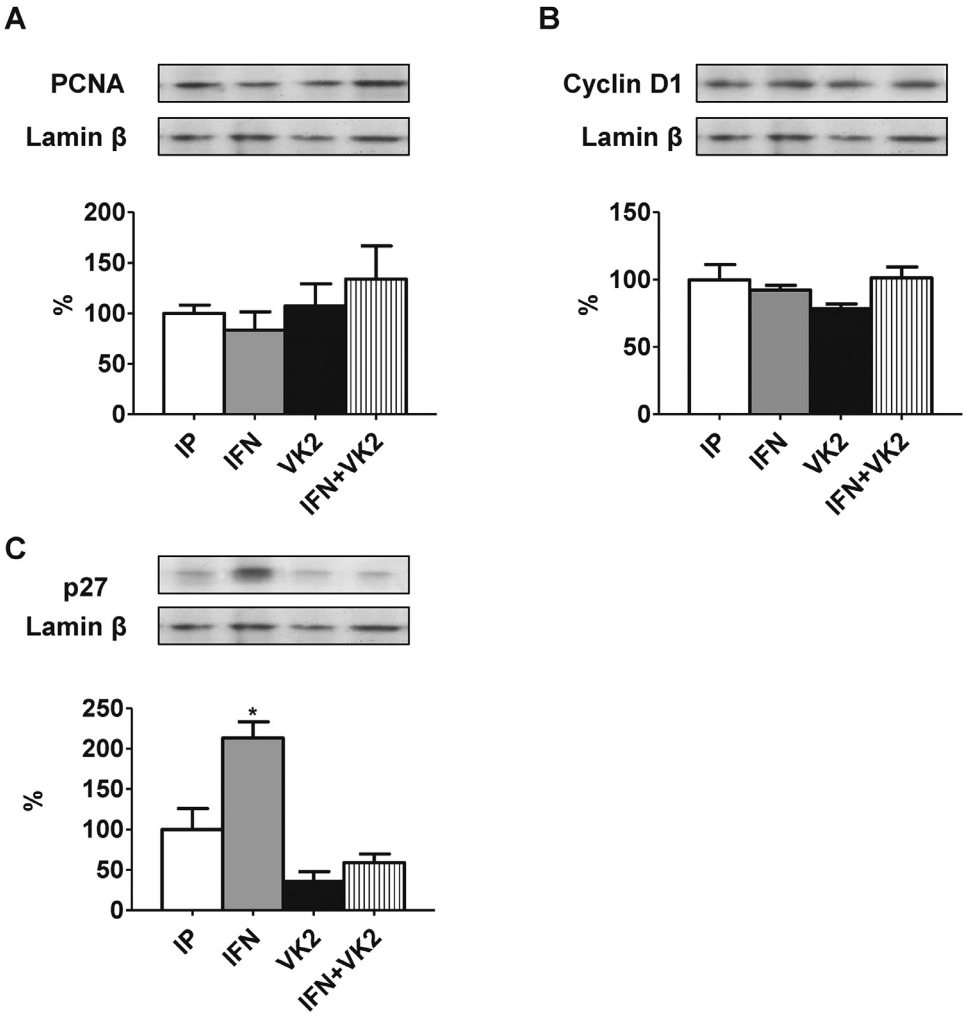


**Fig. 6.** Analysis of cellular proliferation and cell cycle proteins. (**A**) Immunoblotting for PCNA, (**B**) cyclin D1, and (**C**) p27 proteins in liver nuclear fractions. Bars represent mean ± SEM. *$P < 0.05$ vs IP group. IP, initiated-promoted; PCNA, proliferating cell nuclear antigen.

Although it was shown that vitamin $K_2$ induced growth inhibition via cell cycle arrest and apoptosis in a dose-dependent manner for glioma cells in both rat and human cell types [63], in this study we found no changes in cell proliferation in livers from IP rats treated or not with IFN-α-2b, vitamin $K_2$ or both. It was previously shown that vitamin $K_2$ has no effect on cell proliferation inside liver tumors. Some authors claimed that in order to pursue its antiproliferative effects, doses of vitamin $K_2$ should be extremely elevated compared with those used clinically [16]. In a previous report from our group, we found that proliferative index was unchanged after IFN-α-2b administration [21].

It is interesting to note that naturally occurring vitamin K had a poor effect on cell growth and had no effect on the cyclins [64]. Furthermore, vitamin $K_2$ administered with IFN-α in cell lines presented little effect on cell cycle [65]. In this study, however, we showed that vitamin $K_2$ administration in the early stages of liver cancer development had no effect on cell proliferation or apoptosis. Even more, vitamin $K_2$ in combination with IFN-α-2b seemed to block the apoptotic effects of IFN-α-2b through a mechanism linked to an increase in the apoptotic protein Bcl-2. Because both agents are widely used in clinical practice, this combination regimen may be evaluated against HCC in the future.

**Table 1**
PI in AHF

| Group | PI* |
|---|---|
| IP | 331 ± 12.95 |
| IFN | 328.1 ± 5.20 |
| VK2 | 279.3 ± 43.98 |
| IFN + VK2 | 355.5 ± 74.05 |

AHF, altered hepatic foci; IFN, interferon; IP, initiated-promoted; PI, proliferative index; VK2, vitamin $K_2$.
All values represented mean ± SEM.
*PI was expressed as proliferating cells per 1000 hepatocytes.

## Conclusion

No beneficial effects were observed in this study as a result of treating IP rats with vitamin $K_2$, alone or in combination with IFN-α-2b becuase there was no decrease in the number and size of foci, decrease in proliferation, or increased apoptosis. Furthermore, it seems clear that vitamin $K_2$ interferes with IFN-α-2b cellular actions. In this sense, we believe there is an unknown interaction between the signaling pathways of IFN-α-2b and vitamin $K_2$ that leads to the modulation of other signaling pathways with undesired final effects. This interaction might be mediated by the antiapoptotic protein Bcl-2.

Our findings are interesting in many ways. First, this has been, to our knowledge, the only study performed in rats evaluating the effects of IFN-α-2b and vitamin $K_2$ combined therapy on the early stages of liver carcinogenesis. Second, this study highlighted that supportive vitamin supplements/therapies are not always safe, as they could significantly harm patients treated with IFN-α-2b. Finally, administered doses should be studied carefully and be prescribed individually to decrease the risk for secondary effects at a maximum level in patients treated for liver cancer.

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