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Vitamin K3 triggers human leukemia cell death through hydrogen peroxide generation and histone hyperacetylation

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Vitamin K3 (VK3) is a well-known anticancer agent, but its mechanism remains elusive. In the present study, VK3 was found to simultaneously induce cell death, reactive oxygen species (ROS) generation, including superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) generation, and histone hyperacetylation in human leukemia HL-60 cells in a concentration- and time-dependent manner. Catalase (CAT), an antioxidant enzyme that specifically scavenges H_2O_2, could significantly diminish both histone acetylation increase and cell death caused by VK3, whereas superoxide dismutase (SOD), an enzyme that specifically eliminates $O_2^{\bullet -}$, showed no effect on both of these, leading to the conclusion that H_2O_2 generation, but not $O_2^{\bullet -}$ generation, contributes to VK3-induced histone hyperacetylation and cell death. This conclusion was confirmed by the finding that enhancement of VK3-induced H_2O_2 generation by vitamin C (VC) could significantly promote both the histone hyperacetylation and cell death. Further studies suggested that histone hyperacetylation played an important role in VK3-induced cell death, since sodium butyrate, a histone deacetylase (HDAC) inhibitor, showed no effect on ROS generation, but obviously potentiated VK3-induced histone hyperacetylation and cell death. Collectively, these results demonstrate a novel mechanism for the anticancer activity of VK3, i.e., VK3 induced tumor cell death through H_2O_2 generation, which then further induced histone hyperacetylation.

1. Introduction

Previous studies indicate that vitamin K3 (VK3, menadione) is a promising anticancer agent because VK3 not only possesses a splendid anticancer activity itself (Taper et al. 1996; Juan and Wu 1993; Noto et al. 1989; Nutter et al. 1991), but also synergistically exerts anticancer function with lots of conventional chemotherapeutic drugs, such as vitamin C (VC, ascorbic acid, ascorbate) (Nutter et al. 1991; Liao et al. 2000; Gilloteaux et al. 2003; Jamison et al. 2004; Taper et al. 1996, 2004; Verrax et al. 2004; von Gruenigen et al. 2003; Zhang et al. 2001). However, the mechanism for the anticancer activity of VK3 is still unclear.

Previous studies on the mechanism of VK3 anticancer activity indicated that ROS generation plays a critical role in this process (Lamson and Plaza 2003; Brown et al. 1991). This is mainly because VK3 is a quinone, and reduced VK3 (hydroquinone) can be oxidized to semiquinone, then oxidized to VK3 (quinone), by molecular oxygen, coupled with the generation of superoxide anion ($O_2^{\bullet -}$), which may initiate the generation of other ROS, such as hydrogen peroxide (H_2O_2) (Warren et al. 2000; Kachadourian et al. 2001; Saito et al. 2003; Hileman et al. 2004). Coincidently, $O_2^{\bullet -}$ and/or H_2O_2 generation has been found to be involved in the anticancer activity of VK3 in different studies (Nath et al. 1995; Sun et al. 1997; Jamison et al. 2004; Noto et al. 1989). Recent studies disclosed that histone acetylation alteration was differentially involved in the *in vivo* function of ROS. For example, histone hyperacetylation was triggered by ROS during cytokine expression (Ito et al. 2001; Tomita et al. 2003; Gilmour et al. 2002), whereas ROS-induced histone hypoacetylation was involved in Ni^{2+}-triggered tumor cell proliferation arrest and cell death (Kang et al. 2003, 2004a). But up to date, no information is available on whether histone acetylation alteration is also involved in the anticancer activity of VK3 and its-generated ROS.

Histone acetylation plays a pivotal role in the control of eukaryotic gene transcription. This is because in eukaryotes, binding of genomic DNA with histones in the nucleosome prevents the transcription machinery from interacting with promoter DNA sequences, while histone acetylation results in weaker DNA binding, allows access of transcription factors and coactivators to target gene promoter sites, and hence initiates gene transcription (Cheung et al. 2000; Grunstein 1997). The histone acetylation-deacetylation balance is maintained through a balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities in normal cells (Archer and

Abbreviations: VK3, vitamin K3; VC, vitamin C; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; DHE, dihydroethidium; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; HDAC, histone deacetylase; HAT, histone acetyltransferase

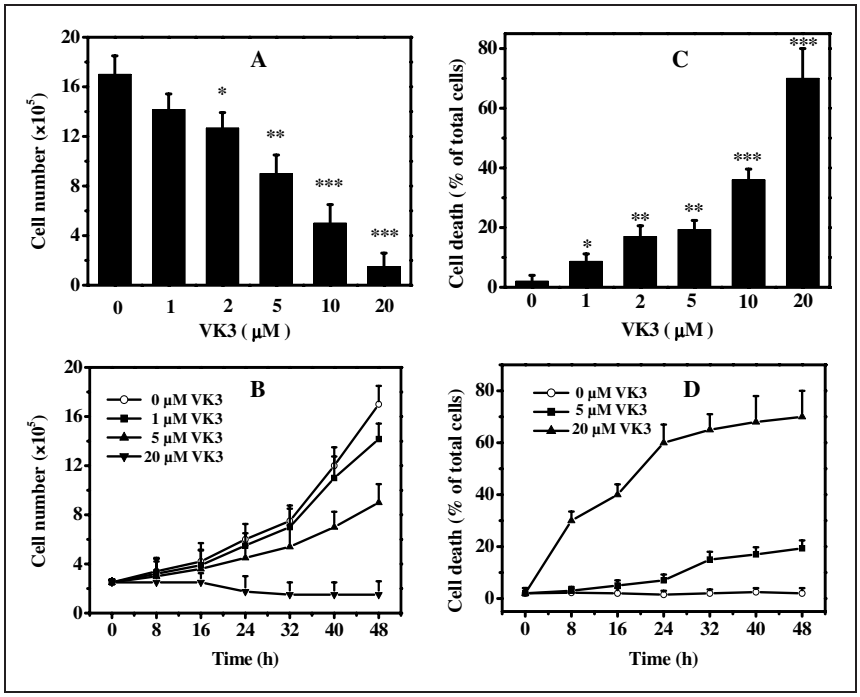

Fig. 1:
Effects of VK3 on the proliferation and viability of HL-60 cells. Treating cells with indicated concentrations of VK3 for 48 h (A or C) or for indicated hours (B or D) decreased the viable cells in HL-60 cells. All data represent mean values of three independent measurements ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus the control group

Hodin 1999; Klochendler-Yeivin and Yaniv 2001), while in tumor cells, this balance favors histone hypoacetylation (Archer and Hodin 1999). Thus development of HDAC inhibitors as anti-cancer drugs has been pursued extensively (Archer and Hodin 1999; Marks et al. 2000; Klochendler-Yeivin and Yaniv 2001; Saito et al. 2003).

Here, we suppose that histone acetylation alteration might be triggered by VK3-induced ROS generation and hence is involved in its anticancer activity. The effects of VK3 on cell viability, ROS generation and histone acetylation were studied in human leukemia HL-60 cells. VK3-treatment simultaneously induced cell death, $O_2^{\bullet -}$ and H_2O_2 generation, and histone hyperacetylation in HL-60 cells, and this histone hyperacetylation was mainly caused by H_2O_2 and played important roles in VK3-induced cell death. These results provide a previously unrecognized mechanism involved in the anticancer activity of VK3, and suggest a new approach, through combination of VK3 with HDAC inhibitors, to synergistically kill cancer cells.

2. Investigations and results

2.1. Anticancer activity of VK3 in human leukemia cells

Treating cells with VK3 concentration- and time-dependently inhibited the proliferation (Fig. 1A and B) and triggered the death of HL-60 cells (Fig. 1C and D).

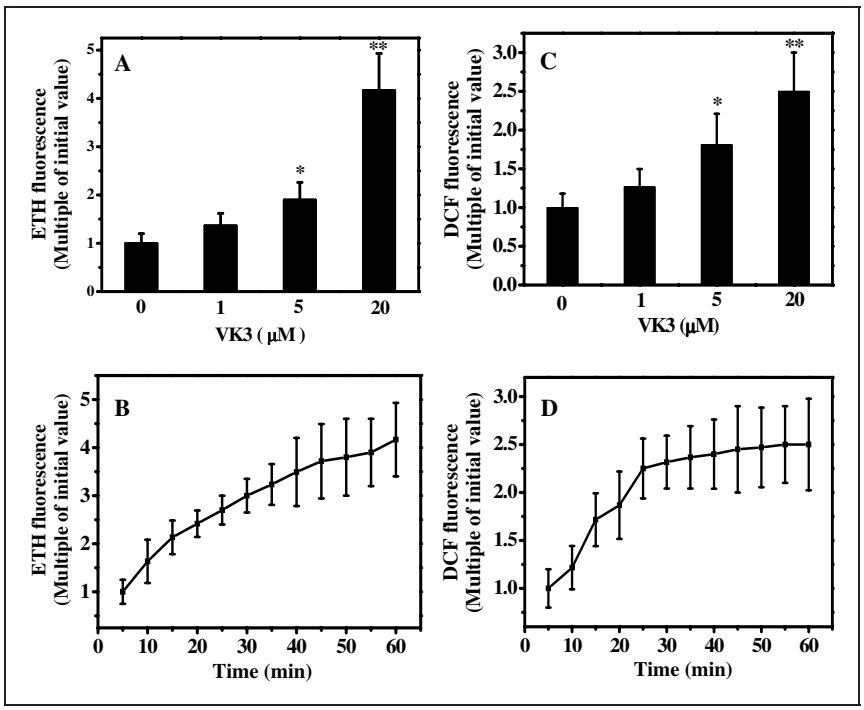

Fig. 2:
Effects of VK3 on ROS generation in HL-60 cells. VK3 induced $O_2^{\bullet -}$ or H_2O_2 generation in HL-60 cells at indicated concentrations for 60 min (A or C) or at 20 μM within 60 minutes (B or D). All data represent mean values of three independent measurements ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, versus the control group

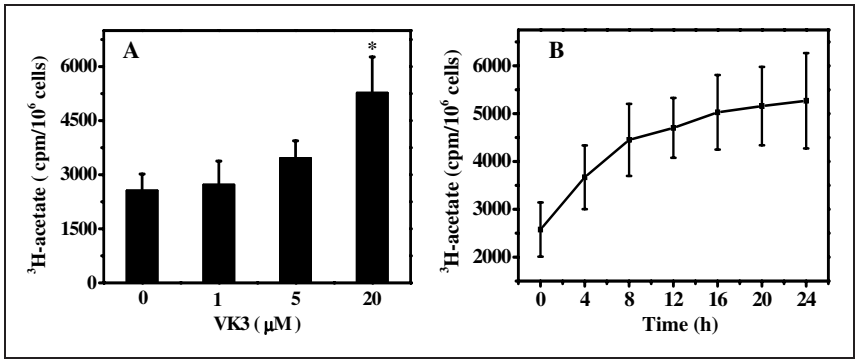

Fig. 3:
Effects of VK3 on histone acetylation in HL-60 cells. Cells were exposed to the indicated concentrations of VK3 for 24 h (A), or 20 μM within 24 hours (B). All data represent mean values of three independent measurements ± SD. $^*p < 0.05$, versus the control group

2.2. *Induction of VK3 on $O_2^{\bullet -}$ and H_2O_2*

ROS generation has been reported in VK3-treated different cells (Juan and Wu 1993; Noto et al. 1989; Nutter et al. 1991; Nath et al. 1995; Sun et al. 1997). To evaluate the generation of ROS in VK3-treated HL-60 cells, fluorescent molecular probes DHE and DCFH-DA were utilized to determine intracellular $O_2^{\bullet -}$ and H_2O_2 generation, respectively. DHE reacts with $O_2^{\bullet -}$ primarily and forms red fluorescent ETH, which binds to DNA and causes amplification of the red fluorescence signal (Vanden Hoek et al. 1997); while DCFH-DA unfixes its diacetate group after entering cells (becoming DCFH) and is oxidized by intracellular H_2O_2 to highly fluorescent DCF (LeBel et al. 1992). As shown in Fig. 2 A and B, VK3-treatment led to a concentration- and time-dependent increase of $O_2^{\bullet -}$ generation in HL-60 cells. When treated with 20 μM VK3, intracellular $O_2^{\bullet -}$ increased continuously and reached to 4.2 folds of that in control group. As shown in Fig. 2C and D, H_2O_2 generation was also triggered in a concentration- and time-dependent manner in VK3-treated HL-60 cells. When treated with 20 μM VK3, intracellular H_2O_2 increased rapidly within first 25 min and reached 2.5 folds at 1 h as compared with the control group.

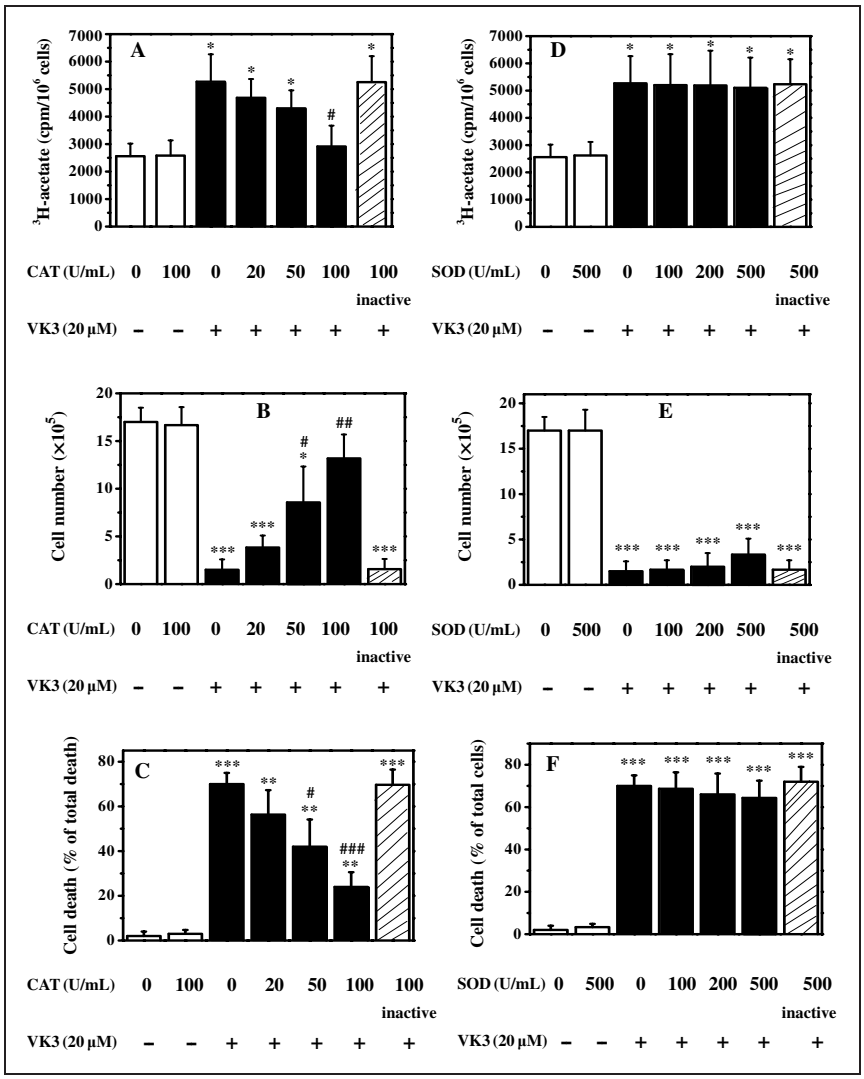

Fig. 4:
Effects of CAT or SOD on VK3-induced histone acetylation (A or D), cell proliferation (B or E), and cell death (C or F). +, − represented adding or not adding the agent, respectively and simultaneously. All data represent mean values of three independent measurements ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus the control group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, versus the 20 μM VK3 alone group

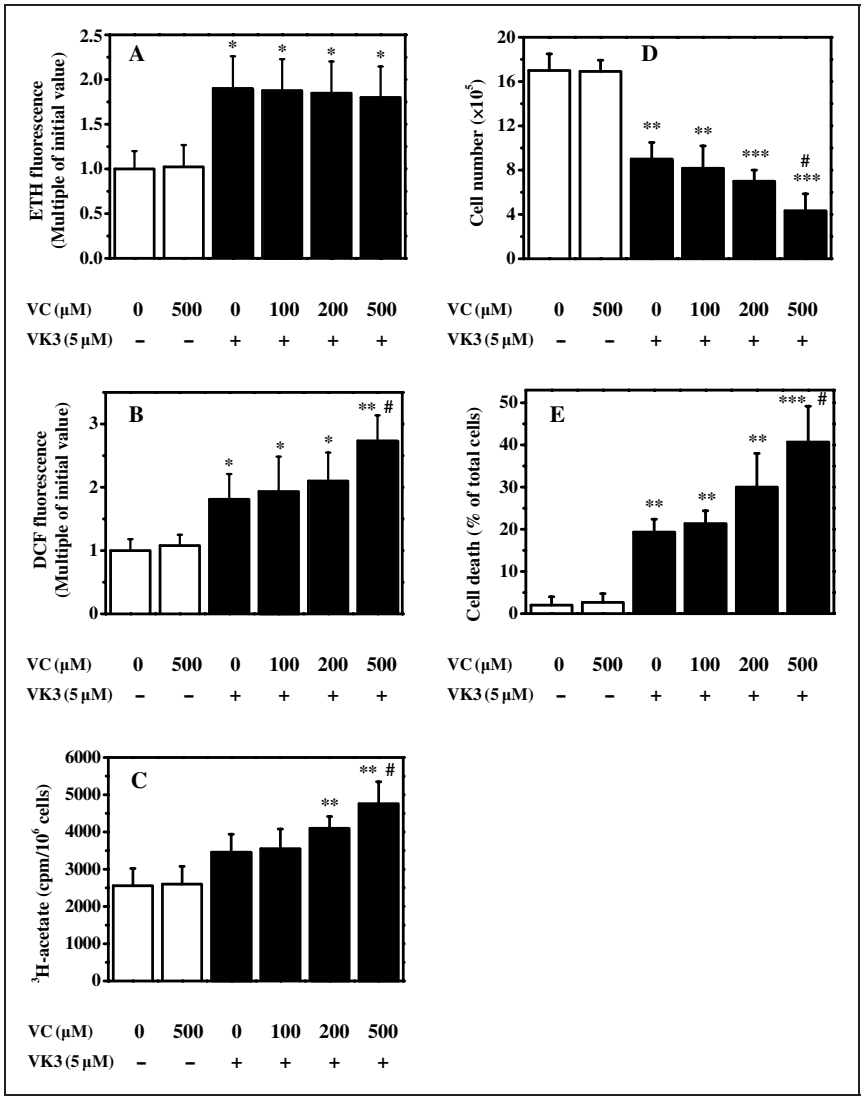

Fig. 5:
Effects of VC on VK3-induced $O_2^{\bullet -}$ (A) or H_2O_2 (B) generation, histone acetylation (C), cell proliferation (D) and cell death (E) in HL-60 cells. +, − represented adding or not adding the agent, respectively and simultaneously. All data represent mean values of three independent measurements ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus the control group. # $p < 0.05$, versus the 5 μM VK3 alone group

2.3. Promotion of VK3 on histone acetylation

Our previous study proved that [^{3}H]-labelled histone acetylation assay and western blotting analysis are equally efficient for evaluating the status of histone acetylation in cultured cells, especially when histones were purified (Kang et al. 2003, 2004a, 2004b), thus [^{3}H]-labelled histone acetylation assay was used in this study. VK3 exposure resulted in a concentration- and time-dependent increase in histone acetylation in HL-60 cells. The remarkable augment was observed at a concentration of 20 μM of VK3 (Fig. 3A), and the increase of histone acetylation was sustained for all tested times (Fig. 3B).

2.4. CAT, but not SOD, attenuated VK3-induced histone hyperacetylation and cell death

Both CAT and SOD are antioxidant enzymes. CAT is able to specifically quench H_2O_2, and SOD specifically scavenges $O_2^{\bullet -}$. In the present study, when 100 U/mL CAT was added into medium, 20 μM VK3-induced histone hyperacetylation (Fig. 4A), cell proliferation arrest (Fig. 4B), and cell death (Fig. 4C) was attenuated, while treating cells with heat-inactivated CAT showed no such effects. But SOD, even at 500 U/mL, could not attenuate those effects of VK3 (Fig. 4D, E and F). These results led to a conclusion that H_2O_2 generation plays an important role in the induction of histone hyperacetylation and cell death by VK3.

2.5. VC enhanced VK3-induced H_2O_2 generation, histone hyperacetylation and cell death

VC potentiated anticancer activity of VK3 through enhancing ROS generation in a wide variety of human cancer cells (Gilloteaux et al. 2003; Jamison et al. 2004; von Gruenigen et al. 2003; Zhang et al. 2001). In the present study, VC showed no effect on VK3-induced $O_2^{\bullet -}$ generation (Fig. 5A), but significantly enhanced H_2O_2 generation (Fig. 5B), histone acetylation (Fig. 5C), cell proliferation arrest (Fig. 5D) and cell death (Fig. 5E) in human leukemia cells, which further confirmed the specific contribution of H_2O_2 generation to VK3-induced histone hyperacetylation and cell death.

2.6. Sodium butyrate enhanced VK3-induced histone hyperacetylation and cell death, but no effect on ROS generation

Sodium butyrate is known to inhibit HDAC activity, leading to histone hyperacetylation in different systems (Riggs et al. 1977; Boffa et al. 1978; Bernhard et al. 1999). In the

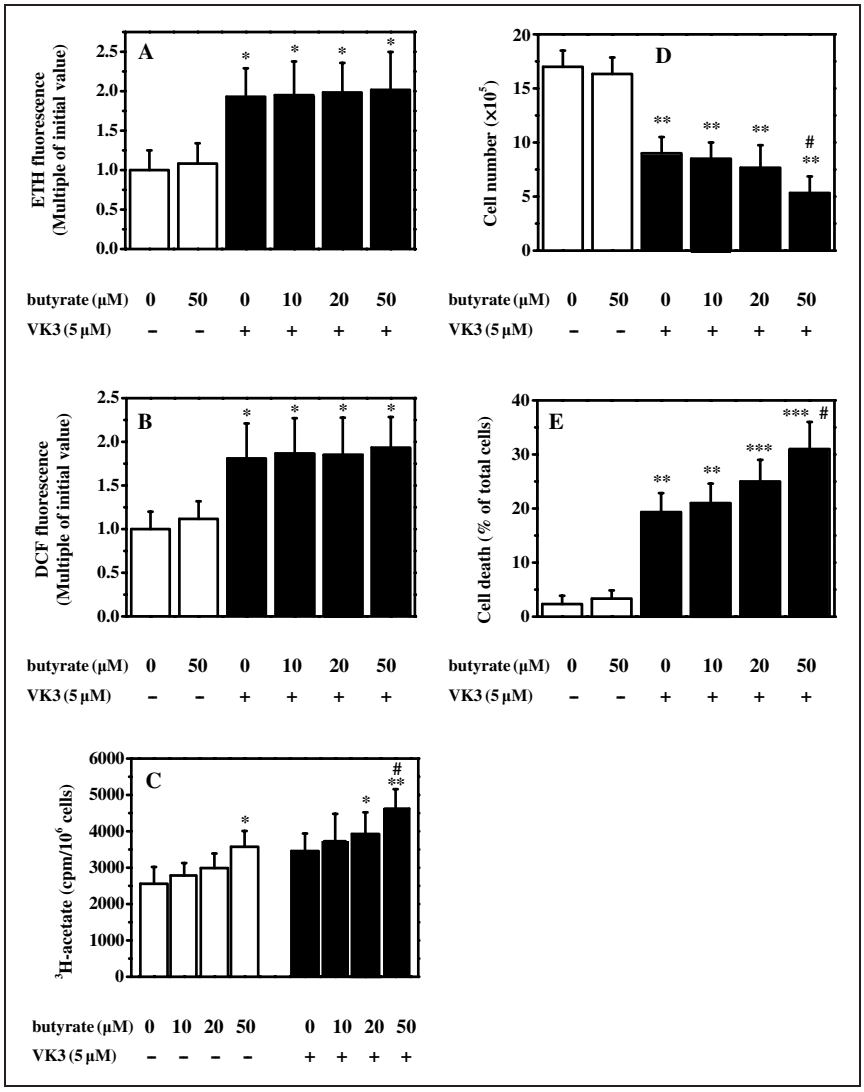

Fig. 6:
Effects of sodium butyrate on VK3-induced $O_2^{\bullet-}$ (A) or H_2O_2 (B) generation, histone acetylation (C), cell proliferation (D) and cell death (E) in HL-60 cells. +, − represented adding or not adding the agent, respectively and simultaneously. All data represent mean values of three independent measurements ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the control group. # $p < 0.05$, versus the 5 μM VK3 alone group

present study, sodium butyrate did not affect VK3-induced $O_2^{\bullet-}$ and H_2O_2 generation (Fig. 6 A and B), but significantly enhanced histone hyperacetylation (Fig. 6C), cell proliferation arrest and cell death (Fig. 6 D and E).

3. Discussion

The anticancer activity of VK3, alone or in combination, has been demonstrated both *in vivo* and *in vitro*, but its mechanism remains unclear. Here we report that histone hyperacetylation resulted from H_2O_2 generation is a novel mechanism involved in the anticancer activity of VK3.

Previous studies showed that $O_2^{\bullet-}$ and H_2O_2 are the main molecules contributing to VK3's anticancer activity (Warren et al. 2000; Nath et al. 1995; Sun et al. 1997; Jamisson et al. 2004; Noto et al. 1989). Reduced VK3 (hydroquinone) has been found to undergo one-electron oxidation and convert the electrons to molecular oxygen, resulting in the generation of $O_2^{\bullet-}$ in cultured cells (Warren et al. 2000). Superoxide, $O_2^{\bullet-}$, can automatically (Lamson and Plaza 2003) or enzymatically (SOD) (Kachadourian et al. 2001; Saito et al. 2003; Hileman et al. 2004) convert to H_2O_2.

Our previous studies indicated that as possible signaling molecules (Kang and Zheng 2004), ROS triggered either histone hyperacetylation or histone hypoacetylation in different situations (Ito et al. 2001; Tomita et al. 2003; Gilmour et al. 2002; Kang et al. 2003, 2004a). Our studies found that diminishing or enhancing H_2O_2 generation by CAT or VC could significantly decrease or increase VK3-induced histone hyperacetylation and cell death, whereas eliminating VK3-generated $O_2^{\bullet-}$ by SOD showed no effects on histone hyperacetylation or cell death. We proved at least that H_2O_2 generation was critically involved in VK3-induced histone hyperacetylation and cell death in HL-60 cells. Consistent with our results, H_2O_2 alone augmented histone acetylation level in other cells (Ito et al. 2001; Gilmour et al. 2002; Tomita et al. 2003). HAT and HDAC are two kinds of enzymes that control histone acetylation modification, and VK3-triggered H_2O_2 generation induced histone hyperacetylation probably through promoting HAT activity and/or inhibiting HDAC activity, due to the fact that H_2O_2 alone are found to increase HAT activity and decrease HDAC activity (Gilmour et al. 2002).

The enhancement or suppression of VK3-induced histone hyperacetylation by regulating H_2O_2 generation also significantly enhanced or suppressed cell proliferation arrest and cell death, suggesting the involvement of histone hyperacetylation in its anticancer activity. Moreover, the enhancement of VK3-induced histone hyperacetylation by sodium butyrate, which did not affect ROS generation, obviously potentiated the anticancer activity of VK3, indicating that histone hyperacetylation indeed is important for

the anticancer activity of VK3, and a combination with HDAC inhibitors maybe one useful strategy to improve the anticancer activity of VK3. Additionally, in VK3-treated cells, VC triggeres ROS generation, and sodium butyrate increases histone acetylation level, which leads to the suggestion that a combination of VC and sodium butyrate is like to more consumingly enhance the anticancer activity of VK3. This probably provides a new approach to design efficient chemotherapy strategies, which combine oxidants, especially H_2O_2 producing agents, with HDAC inhibitors to improve their anticancer activity.

Our results will be more convictive, supposed that the inhibition of histone acetylation attenuates VK3-induced cell proliferation arrest and cell death. Unfortunately, a suitable agent could not be found to inhibit broad range histone acetylation and not to affect ROS generation in VK3-treated cells by this time, though some agents are reported to inhibit histone acetylation (Kang et al. 2003, 2004b; Coull et al. 2002; Balasubramanyam et al. 2004a, b).

In agreement with a previous study (Brinkmann et al. 2001), histone hyperacetylation shoud not be the only mechanism involved in the VK3-induced cell proliferation arrest and cell death. We noticed that 5 μM VK3 triggered ROS generation (both $O_2^{\bullet-}$ and H_2O_2), inhibited cell proliferation and promoted cell death, but did not induce remarkable histone hyperacetylation in HL-60 cells; while 100 U/mL CAT fully restored 20 μM VK3-caused histone acetylation increase, but did not completely block VK3-induced cell proliferation arrest and cell death. These results demonstrated that, besides increasing histone acetylation, VK3 and H_2O_2 might also lead to other molecule events during their induction of cell death. In fact, even at low levels, VK3 is found to efficiently induce DNA strand breaks in human lymphocytes (Woods et al. 1997), and VK3, alone or in combination with VC, induced internucleosomal DNA fragmentation in HL-60 cells due to oxidative stress (Wang et al. 1995).

4. Experimental

4.1. Reagents and chemicals

RPMI 1640 medium was purchased from Gibco Laboratories (Santa Clara, CA, USA) and bovine serum from Si-Ji-Qing Biotechnology Corporation (Hangzhou, China). [^{3}H] acetate was obtained from Beijing Institution of Isotopes. VK3, VC, superoxide dismutase (SOD), catalase (CAT), trypan blue, trichostatin A (TSA), dihydroethidium (DHE), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Corporation. Sodium butyrate was prepared by titrating butyric acid with sodium hydroxide to pH 7.3 (Bernhard et al. 1999). All other reagents are of analytical grade.

4.2. Cell culture and treatment

The HL-60 cells were grown in RPMI 1640 medium supplemented with 10% inactivated bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL, and 2 mM glutamine at 37 °C in 5% CO_2 humidified incubator. VK3 alone or simultaneously combined with other agents, was added into culture medium until cells were harvested. The concentrations and the treated time of VK3 and other agents are indicated in the figures.

4.3. Cell proliferation and viability assay

After seeded in 5 mL culture bottle at a density of 5×10^4 cells/mL, cells were treated with corresponding agents, harvested every 8 h until 48 h and mixed with an equal volume of 0.4% trypan blue solution for 5 min. The stained and unstained cells were counted under a microscope by one investigator. Mean values were obtained from three separate experiments.

4.4. Measurement of intracellular $O_2^{\bullet-}$ and H_2O_2

The level of intracellular $O_2^{\bullet-}$ or H_2O_2 was measured by the alteration of fluorescence resulting from oxidation of dihydroethidium (DHE) to ethidium (ETH) (Miller et al. 1998; Somers et al. 2000), or 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to dichlorofluorescein (DCF) (LeBel et al. 1992). DHE and DCFH-DA was dissolved in DMSO to a final concentration of 20 mM before use. For the measurement of $O_2^{\bullet-}$ or H_2O_2, cells were pre-incubated with 10 μM DHE or DCFH-DA at 37 °C for 30 min, then the excess DHE or DCFH-DA was washed with RPMI-1640 media prior to the treatment with reagents for the indicated time. The intensity of fluorescence was recorded using a fluorescence spectrophotometer, with an excitation filter of 485 nm and an emission filter of 585 nm ($O_2^{\bullet-}$), or with an excitation filter of 485 nm and an emission filter of 535 nm (H_2O_2). The $O_2^{\bullet-}$ or H_2O_2 level was calculated as a ratio: [$O_2^{\bullet-}$] or [H_2O_2] = mean intensity of exposed cells/mean intensity of unexposed cells.

4.5. Histone purification

Preparation of histones from HL-60 cells was done according to Cousens et al. (1979) with the following modifications: the washed cells were suspended in lysis buffer (Cousens et al. 1979) containing trichostatin A (TSA, 100 ng/mL) and phenylmethanesulfonyl fluoride (PMSF, 1 mM). After pipetting up and down 20 times, the nuclei were washed three times in the lysis buffer and once in 10 mM Tris and 13 mM EDTA (pH 7.4). The histones were extracted from the pellet in 0.4 N H_2SO_4. After centrifugation, the histones in the supernatant were collected by cold-acetone precipitation, air-dried, then suspended in 4 M urea and stored at −20 °C before use.

4.6. Histone acetylation assay

Cells were plated at a density of 1×10^6 cells/mL for 24 h and then stimulated with reagents for the indicated times in the presence of 10 μCi/mL [^{3}H] acetate. Then histones were purified as above and [^{3}H] labelled histones were determined by a liquid scintillation counter (Wallac MicroBeta Trilux 1450, Wallac, Turku, Finland).

4.7. Statistical analysis

All data were collected from at least three separate experiments. The statistical analysis for all the experiments in this paper was done using a Student's t-test, and p values less than 0.05, 0.01 or 0.001 were denoted as *, **, or *** respectively.

References

Archer SY, Hodin RA (1999) Histones acetylation and cancer. Curr Opin Genet Dev 9: 171–174.

Balasubramanyam K, Altaf M, Varier RA, Swaminathan V, Ravindran A, Sadhale PP, Kundu TK (2004a) Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. J Biol Chem 279: 33716–33726.

Balasubramanyam K, Varier RA, Altaf M, Swaminathan V, Siddappa NB, Ranga U, Kundu TK (2004b) Curcumin, a novel p300/CBP specific inhibitor of acetyltransferase, represses the acetylation of histones/nonhistone proteins and HAT dependent chromatin transcription. J Biol Chem 279: 51163–51171.

Bernhard D, Ausserlechner MJ, Tonko M, Loffler M, Hartmann BL, Csordas A, Kofler R (1999) Apoptosis induced by the histone deacetylase inhibitor sodium butyrate in human leukemic lymphoblasts. FASEB J 13: 1991–2001.

Boffa LC, Vidali G, Mann RS, Allfrey VG (1978) Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. J Biol Chem 253: 3364–3366.

Brinkmann H, Dahler AL, Popa C, Serewko MM, Parsons PG, Gabrielli BG, Burgess AJ, Saunders NA (2001) Histone hyperacetylation induced by histone deacetylase inhibitors is not sufficient to cause growth inhibition in human dermal fibroblasts. J Biol Chem 276: 22491–22499.

Brown PC, Dulik DM, Jones TW (1991) The toxicity of menadione (2-methyl-1,4-naphthoquinone) and two thioether conjugates studied with isolated renal epithelial cells. Arch Biochem Biophys 285: 187–196.

Cheung WL, Briggs SD, Allis CD (2000) Acetylation and chromosomal functions. Curr Opin Cell Biol 12: 326–333.

Coull JJ, He G, Melander C, Rucker VC, Dervan PB, Margolis DM (2002) Targeted derepression of the human immunodeficiency virus type 1 long terminal repeat by pyrrole-imidazole polyamides. J Virol 76: 12349–12354.

Cousens LS, Gallwitz D, Alberts BM (1979) Different accessibilities in chromatin to histone acetylase. J Biol Chem 254: 1716–1723.

Gilloteaux J, Jamison JM, Arnold D, Taper HS, Von Gruenigen VE, Summers JL (2003) Microscopic aspects of autoschizic cell death in human ovarian carcinoma (2774) cells following vitamin C, vitamin K3 or vitamin C: K3 treatment. Microsc Microanal 9: 311–329.

Gilmour PS, Rahman I, Donaldson K, Macnee W (2002) Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. Am J Physiol 284: L533–L540.

Grunstein M (1997) Histone acetylation in chromatin structure and transcription. Nature 389: 349–352.

Hileman EO, Liu J, Albitar M, Keating MJ, Huang P (2004) Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. Cancer Chemother Pharmacol 53: 209–219.

Ito K, Lim S, Caramori G, Chung KF, Barnes PJ, Adcock IM (2001) Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. FASEB J 15: 1110–1112.

Jamison JM, Gilloteaux J, Nassiri MR, Venugopal M, Neal DR, Summers JL (2004) Cell cycle arrest and autoschizis in a human bladder carcinoma cell line following Vitamin C and Vitamin K3 treatment. Biochem Pharmacol 67: 337–351.

Juan CC, Wu FY (1993) Vitamin K3 inhibits growth of human hepatoma HepG2 cells by decreasing activities of both p34cdc2 kinase and phosphatase. Biochem Biophys Res Commun 190: 907–913.

Kachadourian R, Liochev SI, Cabelli DE, Patel MN, Fridovich I, Day BJ (2001) 2-methoxyestradiol does not inhibit superoxide dismutase. Arch Biochem Biophys 392: 349–353.

Kang J, Zhang Y, Chen J, Chen H, Lin C, Wang Q, Ou Y (2003) Nickel-induced histione hypoacetylation: the role of reactive oxygen species. Toxicol Sci 74: 279–286.

Kang J, Zhang D, Lin C, Chen J, Liu Q, Wang Z (2004a) Involvement of histone hypoacetylation in Ni^{2+}-induced Bcl-2 down-regulation and human hepatoma cell apoptosis. J Biol Inorg Chem 9: 713–723.

Kang J, Lin C, Chen J, Liu Q (2004b) Copper induces histone acetylation through directly inhibiting histone acetyltransferase activity. Chem-Biol Interact 148: 115–123.

Kang J, Zheng R (2004) Dose-dependent regulation of superoxide anion on the proliferation, differentiation, apoptosis and necrosis of human hepatoma cells: the role of intracellular Ca^{2+}. Redox Rep 9: 37–48.

Klochendler-Yeivin A, Moshe Y (2001) Chromatin modifiers and tumor suppression. Biochim Biophys Acta 1551: M1–M10.

Lamson DW, Plaza SM (2003) The anticancer effects of vitamin K. Altern Med Rev 8: 303–318.

LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5: 227–231.

Liao WC, Wu FY, Wu CW (2000) Binary/ternary combined effects of vitamin K3 with other antitumor agents in nasopharyngeal carcinoma CG1 cells. Int J Oncol 17: 323–328.

Marks PA, Richon VM, Rifkind RA (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 92: 1210–1216.

Miller FJ Jr, Gutterman DD, Rios CD, Heistad DD, Davidson BL (1998) Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 82: 1298–1305.

Nath KA, Ngo EO, Hebbel RP, Croatt AJ, Zhou B, Nutter LM (1995) alpha-Ketoacids scavenge H_2O_2 *in vitro* and *in vivo* and reduce menadione-induced DNA injury and cytotoxicity. Am J Physiol 268(1 Pt 1): C227–C236.

Noto V, Taper HS, Jiang YH, Janssens J, Bonte J, De Loecker W (1989) Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K3) treatment on human tumor cell growth *in vitro*. I. Synergism of combined vitamin C and K3 action. Cancer 63: 901–906.

Nutter LM, Cheng AL, Hung HL, Hsieh RK, Ngo EO, Liu TW (1991) Menadione: spectrum of anticancer activity and effects on nucleotide metabolism in human neoplastic cell lines. Biochem Pharmacol 41: 1283–1292.

Riggs MG, Whittaker RG, Neumann JR, Ingram VM (1977) n-Butyrate causes histone modification in HeLa and Friend erythroleukemia cells. Nature 268: 462–464.

Saito M, Sakagami H, Fujisawa S (2003) Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Anticancer Res 23(6C): 4693–4701.

Somers MJ, Mavromatis K, Galis ZS, Harrison DG (2000) Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. Circulation 101: 1722–1728.

Sun JS, Tsuang YH, Huang WC, Chen LT, Hang YS, Lu FJ (1997) Menadione-induced cytotoxicity to rat osteoblasts. Cell Mol Life Sci 53: 967–976.

Taper HS, Keyeux A, Roberfroid M (1996) Potentiation of radiotherapy by nontoxic pretreatment with combined vitamins C and K3 in mice bearing solid transplantable tumor. Anticancer Res 16: 499–503.

Taper HS, Jamison JM, Gilloteaux J, Summers JL, Calderon PB (2004) Inhibition of the development of metastases by dietary vitamin C: K3 combination. Life Sci 75: 955–967.

Tomita K, Barnes PJ, Adcock IM (2003) The effect of oxidative stress on histone acetylation on IL-8 release. Biochem Biophys Res Commun 301: 572–577.

Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB (1997) Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. J Mol Cell Cardiol 29: 2571–2583.

Verrax J, Cadrobbi J, Marques C, Taper H, Habraken Y, Piette J, Calderon PB (2004) Ascorbate potentiates the cytotoxicity of menadione leading to an oxidative stress that kills cancer cells by a non-apoptotic caspase-3 independent form of cell death. Apoptosis 9: 223–233.

von Gruenigen VE, Jamison JM, Gilloteaux J, Lorimer HE, Summers M, Pollard RR, Gwin CA, Summers JL (2003) The in vitro antitumor activity of vitamins C and K3 against ovarian carcinoma. Anticancer Res 23: 3279–3287.

Wang Z, Wang M, Finn F, Carr BI (1995) The growth inhibitory effects of vitamins K and their actions on gene expression. Hepatology 22: 876–882.

Warren MC, Bump EA, Medeiros D, Braunhut SJ (2000) Oxidative stress-induced apoptosis of endothelial cells. Free Radic Biol Med 29: 537–547.

Woods JA, Young AJ, Gilmore IT, Morris A, Bilton RF (1997) Measurement of menadione-mediated DNA damage in human lymphocytes using the comet assay. Free Radic Res 26: 113–124.

Zhang W, Negoro T, Satoh K, Jiang Y, Hashimoto K, Kikuchi H, Nishikawa H, Miyata T, Yamamoto Y, Nakano K, Yasumoto E, Nakayachi T, Mineno K, Satoh T, Sakagami H (2001) Synergistic cytotoxic action of vitamin C and vitamin K3. Anticancer Res 21: 3439–3444.