

## Research Paper

# Silibinin Suppresses Spontaneous Tumorigenesis in $APC^{min/+}$ Mouse Model by Modulating Beta-Catenin Pathway

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**Purpose.** Here we assessed whether silibinin, a nontoxic chemopreventive agent, inhibits spontaneous intestinal tumorigenesis in  $APC^{min/+}$  mouse model, a genetically predisposed animal model of human familial adenomatous polyposis (FAP).

**Materials and Methods.** Six-week-old  $APC^{min/+}$  mice were divided into four groups and orally gavaged with 0.2 ml vehicle, or 250, 500 and 750 mg silibinin/kg body weight in 0.2 ml vehicle for five days/week. After 6 weeks, polyp burden was analyzed and tissues examined for molecular alterations.

**Results.** Silibinin treatments decreased total number of intestinal polyps by 34% ( $P < 0.01$ ), 42% ( $P < 0.01$ ) and 55% ( $P < 0.001$ ), respectively. Immunohistochemical analysis showed that silibinin dose-dependently decreases ( $P < 0.001$ ) proliferation and induces ( $P < 0.001$ ) apoptosis only in intestinal polyps without any considerable effects on normal crypt-villi in  $APC^{min/+}$  or wild-type mice. Further analysis of polyps showed that silibinin decreases  $\beta$ -catenin, cyclin D1, c-Myc and phospho-glycogen synthase kinase-3 $\beta$  expression. Silibinin treatment also decreased phospho-Akt, cyclooxygenase-2, inducible nitric oxide synthase, nitrotyrosine and nitrite levels in polyps, the well-known mediators of intestinal/colon carcinogenesis.

**Conclusion.** Together, these results establish silibinin efficacy in a well-established genetic model of FAP,  $APC^{min/+}$  mouse, and suggest that this natural agent modulates various molecular pathways including  $\beta$ -catenin in its overall chemopreventive efficacy against intestinal carcinogenesis.

**KEY WORDS:** beta-catenin; chemoprevention; colon cancer; COX-2; silibinin.

## INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States (1). Risk factors for CRC are multi-factorial, including a combination of hereditary, dietary and environmental factors (1). Particularly, colon cancer progression is a consequence of accumulation of multiple genetic alterations and mutations. Most notably, mutation in tumor suppressor gene *adenomatous polyposis coli* (APC), which plays a pivotal role in early stages of CRC development by altering  $\beta$ -catenin pathway, is reported in familial and most of the sporadic CRC (2). Recent efforts to control CRC incidence and to minimize

cytotoxicity of chemotherapeutic agents have mainly focused on chemoprevention strategies (3). Since colon carcinogenesis is a multistage process involving progression of normal epithelium to malignant phenotype that exhibits various alterations in cellular and molecular signaling cascades (2), chemoprevention by natural products is expected to delay neoplastic promotion/progression to more advanced malignancy (3). However, the efficacy of chemopreventive agents needs to be established in well-defined preclinical models of CRC before embarking on to clinical trials.

$APC^{min/+}$  mouse is one of the CRC animal models which harbors dominant germline mutation at codon 850 of the APC, the mouse homologue of human APC gene (4).  $APC^{min/+}$  mice are heterozygous for a mutant allele of APC that has lost its function by loss of heterozygosity (5).  $APC^{min/+}$  mice develop multiple adenomas primarily in their small intestine, and only fewer tumors arise in the colon, mostly due to aberrant  $\beta$ -catenin signaling (4,5). Intestinal polyps in  $APC^{min/+}$  mice are similar to those in duodenum of familial adenomatous polyposis (FAP) patients (6), suggesting that  $APC^{min/+}$  mouse represents a clinically relevant animal model mimicking human disease. This mouse model is indeed extensively used to study molecular mechanisms involved in intestinal tumorigenesis and cancer chemopreventive efficacy of different agents (6,7).

Silibinin, a flavonolignan constituent in silymarin extracted from milk thistle (*Silybum marianum*) plant (8),

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**ABBREVIATIONS:** APC, Adenomatous polyposis coli; COX-2, cyclooxygenase-2; CRC, colorectal cancer; FAP, familial adenomatous polyposis; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; IHC, immunohistochemistry; iNOS, inducible nitric oxide synthase.

has received significant attention in recent years due to its strong cancer chemopreventive and anti-cancer efficacy against a wide-range of epithelial cancers (9–20). Recent studies by others and we have shown silibinin efficacy in various *in vitro* and *in vivo* experimental models of skin (9), lung (11,12), prostate (10,13,14) and bladder (15,16) cancers. Regarding CRC, *in vitro* studies showed that silibinin causes cell cycle arrest and induces apoptosis in human CRC cells (17), and that its oral feeding to nude mice inhibits HT-29 xenograft growth by inhibiting tumor cell proliferation and angiogenesis (18). Regarding its CRC chemopreventive efficacy, we showed that silibinin feeding inhibits azoxymethane (AOM)-induced colonic aberrant crypt foci formation in Fisher 344 rats (19). Kohno *et al.* (20) showed that crude extract of milk thistle—namely silymarin—prevents AOM-induced colon carcinogenesis in rats. More recently, 0.2% silibinin feeding in diet to *APC<sup>min/+</sup>* mice starting at 11 weeks of age for 7 weeks is shown to produce marginal efficacy against gastrointestinal carcinogenesis, which may be due to the fact that silibinin administration was started at later age (21). The present study, therefore, was designed using three different doses of silibinin (250, 500 and 750 mg/kg body weight) gavage feeding to *APC<sup>min/+</sup>* mice starting at 6 weeks of age. This study addresses whether this agent inhibits intestinal polyposis in *APC<sup>min/+</sup>* mice, and, if it does, what potential mechanisms of its efficacy towards  $\beta$ -catenin pathway and other molecular regulators are known to be involved in tumorigenesis in *APC<sup>min/+</sup>* mouse model.

## MATERIALS AND METHODS

### Animals and Treatment Protocol

Heterozygous male Min (C57BL/6J-*APC<sup>min/+</sup>*) and wild-type C57BL/6J male mice (5 weeks old) were purchased from Jackson Laboratory. Silibinin was from Sigma, and its purity was checked as >98% as described earlier (22). After one week of acclimatization, *APC<sup>min/+</sup>* mice (6-week old) were divided into four groups of 12 animals each. Group 1 was given 0.2 ml vehicle consisting of 0.5% carboxymethyl cellulose and 0.025% Tween 20 in distilled water. Group 2–4 animals received 250, 500 and 750 mg silibinin/kg body weight, respectively, by oral gavage in 0.2 ml vehicle for 5 days/week for 6 weeks. Two negative control groups ( $n=12$ /group) of wild-type C57BL/6J mice were given vehicle (Group 5) or 500 mg silibinin/kg body weight (Group 6) as for *APC<sup>min/+</sup>* mice. Mice in all groups received AIN-76A pellet diet and water *ad libitum* throughout experimental period. Food consumption and body weights of mice in all groups were recorded weekly, and animals were monitored regularly for general health. Animal care and treatments were in accordance with approved protocol, adhered to the “principles of laboratory animal care” and were approved by IACUC.

The rationale for selecting these silibinin doses (250, 500 and 750 mg/kg bw) was based on recent publications by us and others (12–14,19,23). These silibinin doses (250–750 mg/kg bw/day) have been extrapolated from the mice consuming up to 1% w/w silibinin in diet without any apparent toxicity, which we have shown in many animal studies (12–14). In the present study, the highest dose of

silibinin (750 mg/kg bw/day) corresponds to 0.5% w/w silibinin in diet (average mouse body weight 20 g; average diet consumption 3 g/mouse/day or 15 mg silibinin/mouse/day) (13,14).

### Necropsy

After 6 weeks of treatment (at 12 weeks of age), all animals were sacrificed, blood was collected in heparinized tubes by cardiac puncture, and plasma was separated by centrifugation and stored at  $-80^{\circ}\text{C}$ . Small and large intestinal tracts were removed, opened longitudinally and rinsed with saline. The small intestinal samples were divided by length into three equal segments (proximal, middle and distal) and spread onto microscope slides. The total number and size of polyps in all intestinal and colonic segments were examined under a dissecting microscope and measured with digital caliper. A portion of small intestinal and colonic tissues were fixed in 10% (v/v) phosphate-buffered formalin for histopathology and immunohistochemical (IHC) analyses. Tissue sections (5  $\mu\text{m}$ ) were cut, processed, and stained with H&E for histopathological evaluation. Remainder of the small intestine and colon samples were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Immunohistochemistry and Quantification of Immunostaining

Paraffin-embedded sections (5- $\mu\text{m}$  thick) were deparaffinized and stained using specific primary antibodies followed by 3,3-diaminobenzidine (DAB) staining, as previously described (14,18). Primary antibodies used were anti-PCNA (1:250 dilution; Dako), anti- $\beta$ -catenin (1:100 dilution; Santa-Cruz), anti-nitrotyrosine (1:100 dilution; SantaCruz), anti-c-Myc (1:50 dilution; SantaCruz), rabbit polyclonal anti-cyclin D1 (1:100 dilution; Neomarkers), anti-inducible nitric oxide synthase (iNOS) (1:100 dilution, Abcam), anti-phosphoglycogen synthase kinase (GSK)-3 $\beta$  (Ser 9) (1:50 dilution, Cell Signaling), anti-phospho-Akt (1:50 dilution, Cell Signaling), and goat polyclonal anti-COX-2 (1:100 dilution; Santa-Cruz). Biotinylated secondary antibodies used were rabbit anti-mouse IgG (Dako) and goat anti-rabbit IgG (Santa Cruz Biotechnology). Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using Dead End Colorimetric TUNEL System (Promega Corp). In addition to polyps, all IHC studies also included the crypt and villous regions from all three portions of small intestine. Positive cells were quantified by counting brown-stained cells among total number of cells at 5 randomly selected fields at 400X magnification. iNOS, nitrotyrosine and COX-2 were quantified by immunoreactivity (represented by intensity of brown staining) and scored as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong).

### Assay of Nitrite/Nitrate Levels in Mouse Plasma

iNOS activity in plasma was measured using nitric oxide production by estimating nitrate and nitrite after complete conversion of nitrate into nitrite by nitrate reductase employing nitrate/nitrite colorimetric assay kit (R&D systems). The

final nitrite concentration was the sum of nitrite plus reduced nitrate in each sample and was calculated as an index of iNOS activity as described previously (24).

### Statistical and Microscopic Analyses

Statistical analyses were done using SigmaStat software version 3.5 (Jandel Scientific). Quantitative data are presented as mean±SEM. Statistical significance of differences between control and silibinin-fed groups was determined by unpaired Student's *t*-test, and  $P < 0.05$  was considered statistically significant. Microscopic IHC analyses were done by Zeiss Axioskop 2 microscope (Carl Zeiss, Inc.) and photomicrographs were captured by Carl Zeiss AxioCam MRC5 camera.

## RESULTS

### Silibinin Feeding Prevents Spontaneous Intestinal Polyposis in $APC^{min/+}$ Mice

At the end of the study (12 weeks of age), control  $APC^{min/+}$  mice developed an average of 20–48 polyps in small intestine and ~0–4 polyps in colon. Six weeks of silibinin treatment, however, decreased total number of intestinal polyps by 34% ( $P < 0.01$ ), 42% ( $P < 0.01$ ) and 55% ( $P < 0.001$ ) at 250, 500 and 750 mg/kg body weight doses, respectively (Fig. 1A). Although silibinin at three dose levels significantly reduced the total number of polyps in small intestine, this effect (~50% decrease) was statistically non-significant (due to a fewer number of polyps) in colon (Fig. 1B). Importantly, all silibinin treatments significantly reduced number of polyps in proximal, middle and distal portions of small intestine, which was specifically prominent at 750 mg/kg dose where number of polyps was reduced by 70% ( $P < 0.001$ ), 48% ( $P < 0.001$ ), and 51% ( $P < 0.001$ ), respectively (Fig. 1C). In wild-type C57BL/6J mice, both control and silibinin-fed groups did not show any polyps throughout the intestine (data not shown).

In size distribution analysis of small intestinal polyps, silibinin at 500 and 750 mg/kg doses reduced the occurrence/development of <1 mm diameter size polyps by 38% ( $P < 0.001$ ) and 48% ( $P < 0.001$ ) and of 1–2 mm diameter size polyps by 39% ( $P < 0.001$ ) and 52% ( $P < 0.001$ ), respectively (Fig. 1D). Lowest silibinin dose (250 mg/kg) also showed statistically significant inhibition (Fig. 1D). Silibinin at 250, 500 and 750 mg/kg doses reduced the occurrence of 2–3 mm diameter size polyps by 44% ( $P < 0.05$ ), 49% ( $P < 0.01$ ) and 69% ( $P < 0.001$ ), respectively, and more importantly caused a complete suppression ( $P < 0.05$ ) of the occurrence of >3 mm diameter size polyps (1.08±0.6 polyps in control *versus* no polyps in all three silibinin-treated groups) (Fig. 1D). In colon, these silibinin doses decreased the number of larger polyps by 50% ( $P > 0.05$ ), 67% ( $P < 0.05$ ) and 75% ( $P < 0.05$ ) for 1–2 mm size, respectively, and 24–76% ( $P > 0.05$ ) for >2–3 mm size; there were no polyps beyond 3 mm size (Fig. 1B). Collectively, these results clearly showed that silibinin treatment strongly and dose-dependently reduces the number as well as growth of polyps in intestine of  $APC^{min/+}$  mice.

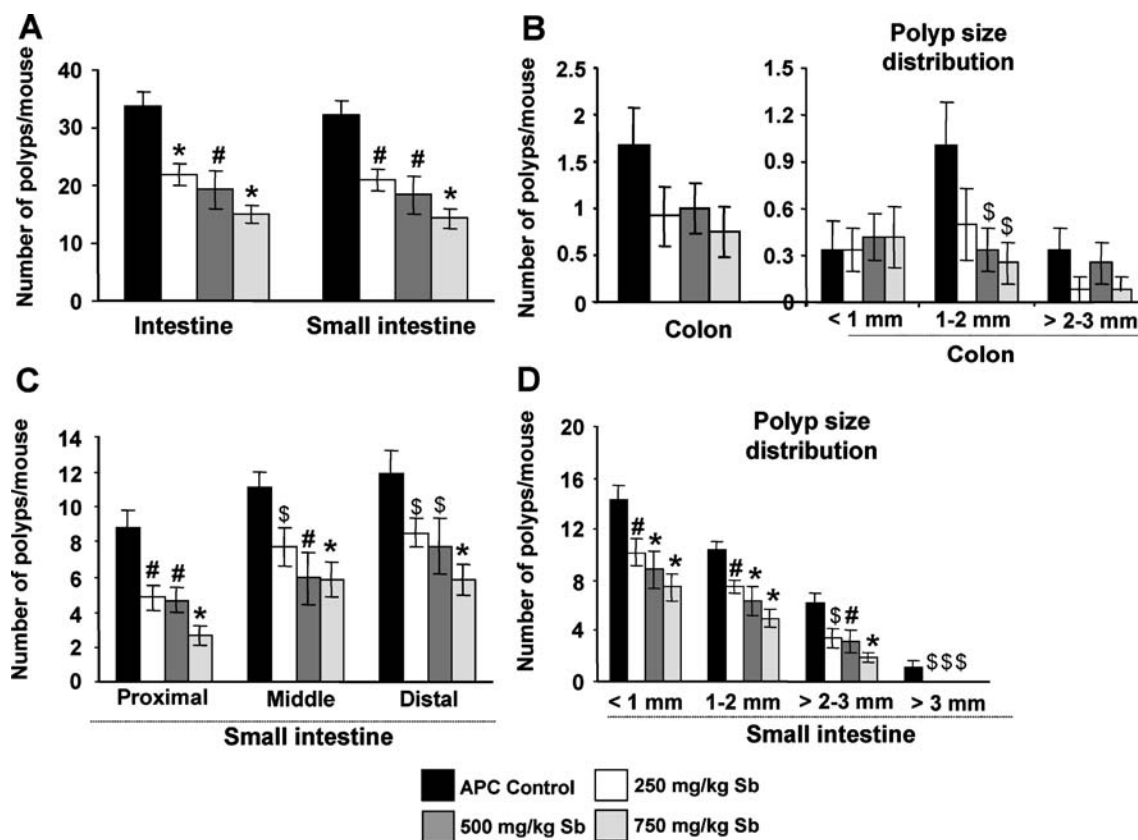
### Effects of Silibinin on Histopathology of Intestine and Gross Toxicity

In  $APC^{min/+}$  mice, all polyps were histologically identified as adenomas (Fig. 2A); however, the number and diameter of adenomas were reduced in silibinin-administered animals as described above. We did not observe any other histological changes in the crypt-villus axis between control and silibinin-treated  $APC^{min/+}$  mice (Fig. 2B), which was also histologically similar to that of wild-type mice (Fig. 2C). Further, control and silibinin-treated wild-type C57BL/6J mice showed normal intestinal histology (Fig. 2C). During the experiment, all mice were monitored for body weight and diet consumption which could be either due to intestinal obstruction caused by polyps or silibinin treatment. Food consumption and body weight gain did not show any considerable change between control and silibinin groups throughout the study (Fig. 2D), and no mortality was observed in any group. Silibinin treatment also did not produce any signs of toxicity or any gross changes indicative of toxicity in the organs (liver, lung and kidney) examined. Together, these results suggest that silibinin treatment exerts significant chemopreventive effect selectively against intestinal polyps.

### Silibinin Treatment Reduces Proliferation But Induces Apoptosis Selectively In Small Intestinal Polyps of $APC^{min/+}$ Mice

To assess *in vivo* antiproliferative effect of silibinin, proximal, middle and distal segments of small intestine were analyzed immunohistochemically for PCNA, a marker for cell proliferation (25). Microscopic examination of tissue sections showed a decreased number of PCNA-positive cells only in the polyps from silibinin-treated animals compared to  $APC^{min/+}$  controls (data not shown). Quantification of PCNA staining showed that silibinin treatment decreases PCNA-positive cells by 20–27% in proximal, 22–36% in middle and 30–42% in distal small intestinal polyps at 250 and 500 mg/kg dose levels compared to  $APC^{min/+}$  controls (Fig. 3A). A much stronger inhibitory effect of silibinin was observed at its highest dose (750 mg/kg), which reduced proliferation index by 45% ( $P < 0.001$ ), 48% ( $P < 0.001$ ) and 54% ( $P < 0.001$ ) in the polyps in proximal, middle and distal small intestinal segments, respectively (Fig. 3A). Together, these results clearly suggest an *in vivo* anti-proliferative effect of silibinin selectively in the polyps during spontaneous intestinal tumorigenesis in  $APC^{min/+}$  mice, as we did not observe any difference in proliferation indices in crypt and villus regions of all three small intestinal segments from control and silibinin-treated  $APC^{min/+}$  and wild-type C57BL/6J mice (Fig. 3A and B).

To assess the effects of silibinin on *in vivo* apoptosis induction, we next assessed apoptotic index by TUNEL staining. Qualitative microscopic examination of TUNEL-stained intestinal sections showed an increase in TUNEL-positive cells only in the polyps from silibinin-treated  $APC^{min/+}$  mice compared to  $APC^{min/+}$  controls (data not shown). Quantification of TUNEL staining showed that silibinin treatment increases apoptotic cell population by 1.5, 1.9 and 2.0 folds in proximal, by 1.8, 3.0 and 3.0 folds in middle, and by 2.0, 3.0 and 4.0 folds in distal small intestinal polyps at 250, 500 and 750 mg/kg dose levels,



**Fig. 1.** Silibinin feeding prevents spontaneous intestinal polyposis in *APC<sup>min/+</sup>* mice. At the end of the study detailed in Materials and Methods, intestinal segments were examined for total number of polyps as well as their sizes. Number of polyps/mouse in *APC<sup>min/+</sup>* mice is shown for (A) whole intestinal tract and small intestine and (B) large intestine with polyp size distribution (<1 mm, 1–2 mm and >2–3 mm) in colon having no polyps larger than 3 mm. C, number of polyps/mouse in proximal, middle and distal portions of small intestine. D, size distribution (<1 mm, 1–2 mm, >2–3 mm and >3 mm) of polyps in small intestine. The bars shown in each case are mean±SEM of 12 animals in each group. Significance was analyzed by unpaired Student's *t*-test. \$, *P*<0.05; #, *P*<0.01; \*, *P*<0.001 versus control; Sb, silibinin.

respectively, over that of *APC<sup>min/+</sup>* controls (Fig. 3C). Similar to PCNA, we did not observe any difference in apoptotic indices in the crypt and villus regions of all three small intestinal segments from control and silibinin-treated *APC<sup>min/+</sup>* and wild-type C57BL/6J mice (Fig. 3C and D). Overall, these results suggest an important role of pro-apoptotic effect of silibinin in suppressing polyp development in *APC<sup>min/+</sup>* mice, which could be another potential mechanism underlying its chemopreventive effect on intestinal tumorigenesis in *APC<sup>min/+</sup>* mice. Since our further IHC analysis showed no significant changes in the expression of various molecular markers in the crypt and villus regions throughout the small intestine of control and silibinin-treated *APC<sup>min/+</sup>* mice, the results of further analyses in these normal tissues or wild-type mice are not discussed in subsequent sections.

**Silibinin Treatment Reduces Phospho-Akt Levels Selectively in Small Intestinal Polyps of *APC<sup>min/+</sup>* Mice**

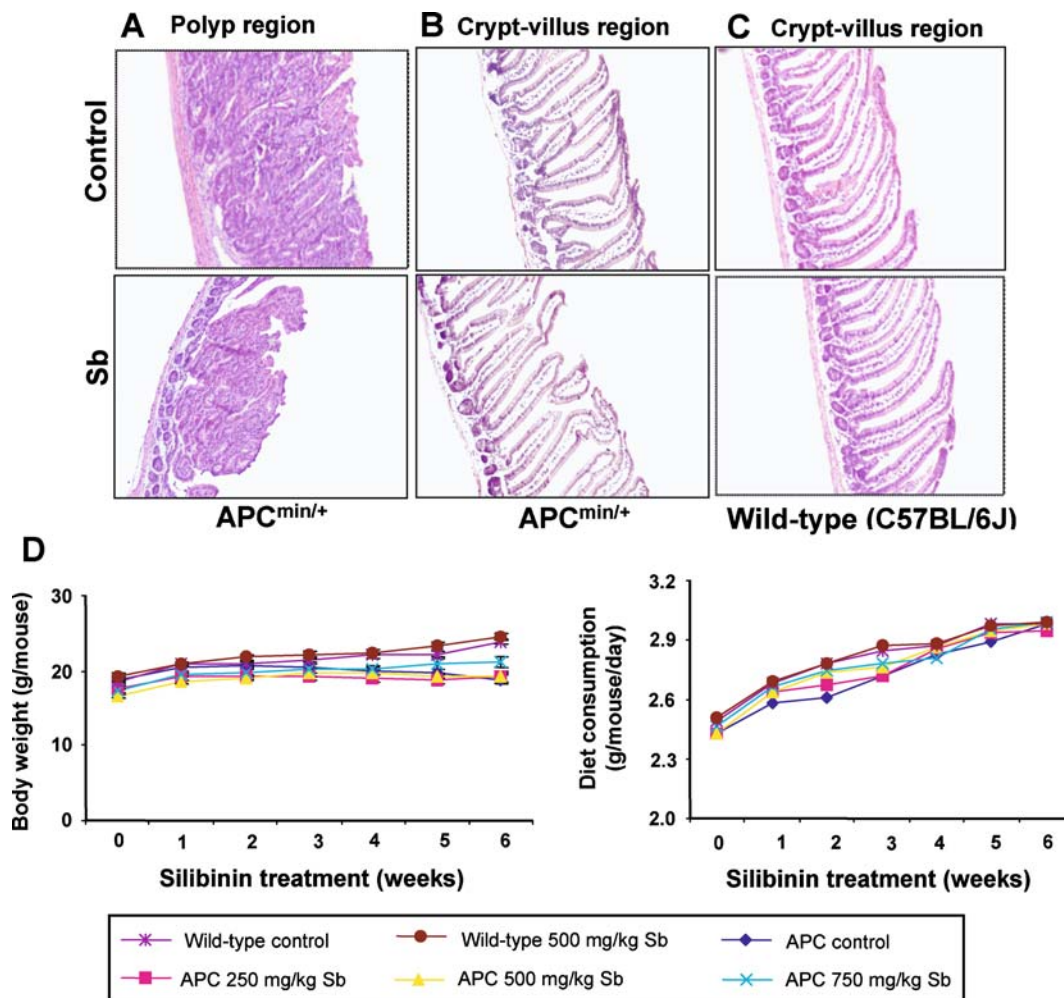
Akt is known to activate various molecules involved in cell cycle progression, cellular growth and survival, and its phosphorylation represents activation state during carcinogenesis (26). To identify silibinin effect on Akt activation, we examined phospho-Akt expression by IHC. Microscopic observation of all three small intestinal segments indicated

strong phospho-Akt-positive cells in the polyps of *APC<sup>min/+</sup>* controls, which decreased strongly with silibinin treatments (Fig. 4A). The quantification of phospho-Akt-positive cells showed a reduction by 23–28% in proximal, 28–44% in middle and 35–53% in distal portions of small intestinal polyps in silibinin-treated samples compared to *APC<sup>min/+</sup>* controls (Fig. 4A).

**Silibinin Treatment Modulates Aberrant β-catenin Pathway Selectively in Small Intestinal Polyps of *APC<sup>min/+</sup>* Mice**

Alteration in β-catenin pathway due to loss of *APC* function is implicated in CRC initiation and progression (27–29). To assess silibinin effect on β-catenin pathway, we analyzed by IHC the expression of β-catenin together with other molecular players associated with this pathway, including phospho-GSK-3β, cyclin D1 and c-Myc in all three small intestinal segments. Microscopic examination of tissue sections showed increased numbers of β-catenin, phospho-GSK-3β, cyclin D1 and c-Myc-positive cells in the polyps of control *APC<sup>min/+</sup>* mice, which were substantially decreased by silibinin treatment (Fig. 4B–E). Quantification of immunostained sections showed that silibinin dose-dependently decreases β-catenin-positive cells by 15–40% (*P*<0.01–0.001) in proximal, 18–43% (*P*<0.001) in middle, and 24–52% (*P*<0.001) in distal





**Fig. 2.** Silibinin feeding selectively prevents polyp burden in small intestine of  $APC^{min/+}$  mice without any apparent toxicity. At the end of the study detailed in Materials and Methods, small intestine from wild-type C57BL/6J and  $APC^{min/+}$  mice was processed for H&E staining followed by histological evaluation. *A*, control and silibinin-treated polyp area from small intestine of  $APC^{min/+}$  mice (X100). Normal crypt-villus axis from (*B*) control and silibinin (750 mg/kg bw)-treated  $APC^{min/+}$  mice, and (*C*) control and silibinin (500 mg/kg bw)-treated wild-type C57BL/6J mice (X100). During experiment, (*D*) body weight (gram/mouse) and diet consumption (gram/mouse/day) were monitored. Sb, silibinin.

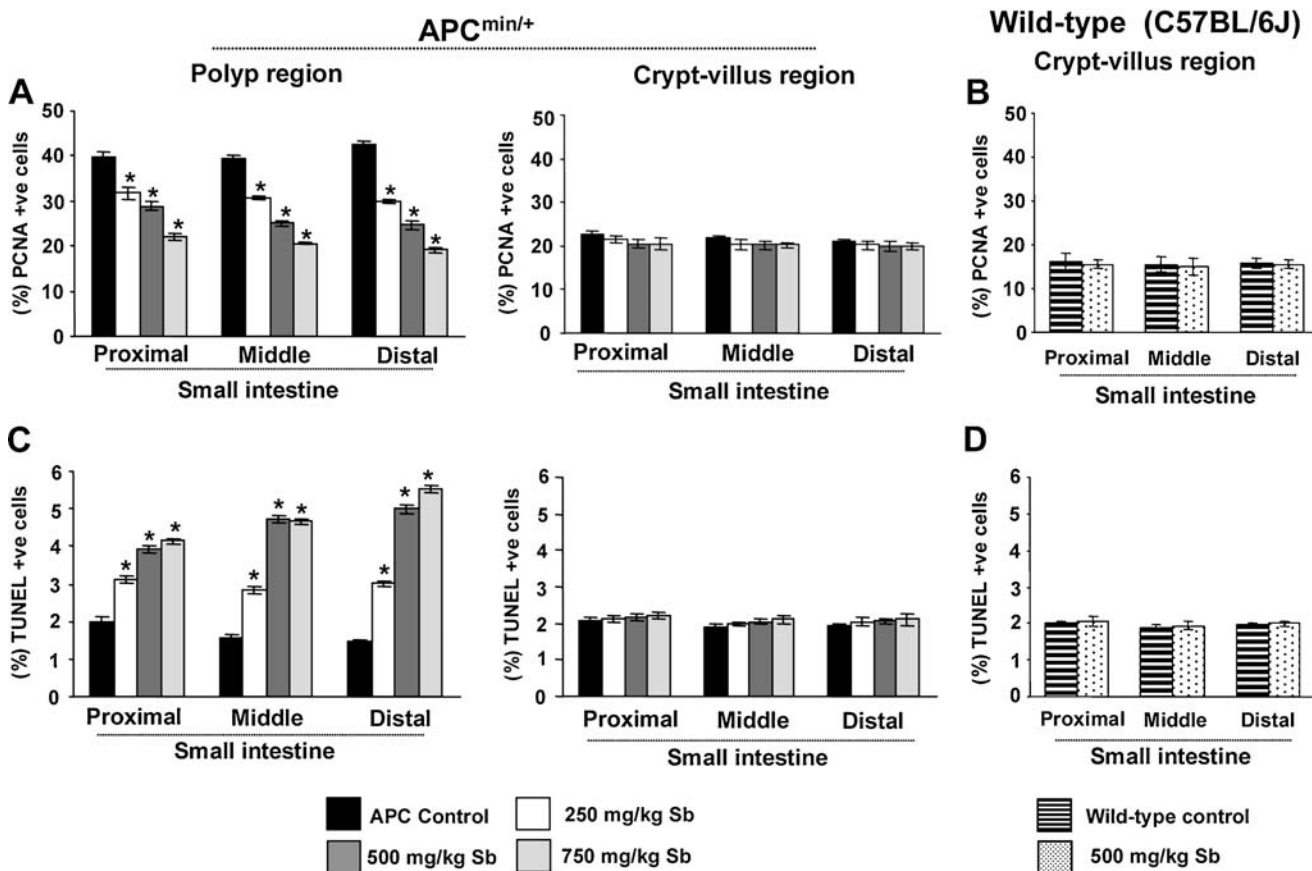
segments of small intestinal polyps (Fig. 4B). Phosphorylation of GSK-3 $\beta$  at serine 9 residue down-regulates its activity and subsequently leads to both cytoplasmic stabilization and increased nuclear translocation of  $\beta$ -catenin (30). In our study, quantification of phospho-GSK-3 $\beta$  (ser 9) staining showed that silibinin treatment (250–750 mg/kg) reduces phospho-GSK-3 $\beta$ -positive cells by 22–44% ( $P < 0.01$ –0.001), 24–48% ( $P < 0.001$ ) and 30–52% ( $P < 0.001$ ) in the polyps in proximal, middle and distal portions of small intestine, respectively (Fig. 4C).

Both cyclin D1 and c-Myc are downstream transcriptional targets of  $\beta$ -catenin pathway (31,32), and are implicated in initiation of intestinal tumorigenesis (29). Over-expression of cyclin D1 and c-Myc in tumor cells is frequently common and positively correlates with proliferative index (29). In the present study, compared to control  $APC^{min/+}$  mice, we observed the reduction in cyclin D1-positive cells by 20–49% ( $P < 0.01$ –0.001) in proximal, 25–50% ( $P < 0.001$ ) in middle, and 33–52% ( $P < 0.001$ ) in distal portions of small intestinal polyps by silibinin treatments (Fig. 4D). Silibinin treatments also decreased c-Myc-

positive cells by 23–41% ( $P < 0.001$ ) in proximal, 25–49% ( $P < 0.001$ ) in middle, and 29–50% ( $P < 0.001$ ) in distal portions of small intestinal polyps (Fig. 4E).

#### Silibinin Treatment Decreases COX-2 and iNOS Expression Selectively in Small Intestinal Polyps of $APC^{min/+}$ Mice

COX-2 and iNOS have been strongly implicated in both colon and small intestinal carcinogenesis (33). Accordingly, we next studied silibinin effect on COX-2 and iNOS expression by IHC. Our results showed strong immunoreactivity for both COX-2 and iNOS in small intestinal polyps of  $APC^{min/+}$  mice, which decreased strongly by silibinin treatments (IHC staining data are not shown). Quantification of immunostained-cells showed that silibinin, particularly at 750 mg/kg dose, decreases COX-2 and iNOS levels ( $P < 0.01$ –0.001) by 38% and 40% in proximal, 36% and 41% in middle, and 50% and 51% in distal portions of small intestinal polyps, respectively (Fig. 5A and B). Together, these results indicate that COX-2 and iNOS



**Fig. 3.** Silibinin treatment reduces proliferation but induces apoptosis selectively in small intestinal polyps of *APC<sup>min/+</sup>* mice. At the end of the study detailed in Materials and Methods, small intestinal segments were processed for PCNA and TUNEL staining. The quantitative data for (A,B) PCNA and (C,D) TUNEL are shown for intestinal polyps and crypt-villus region of *APC<sup>min/+</sup>* mice (A and C) and crypt-villus region of C57BL/6J wild type mice (B and D), respectively. Data represent mean±SEM of 8 animals in each group in each case. \*, *P*<0.001 versus control; Sb, silibinin.

could be potential molecular targets for the chemopreventive effects of silibinin.

**Silibinin Treatment Decreases iNOS Activity in Small Intestine of *APC<sup>min/+</sup>* Mice**

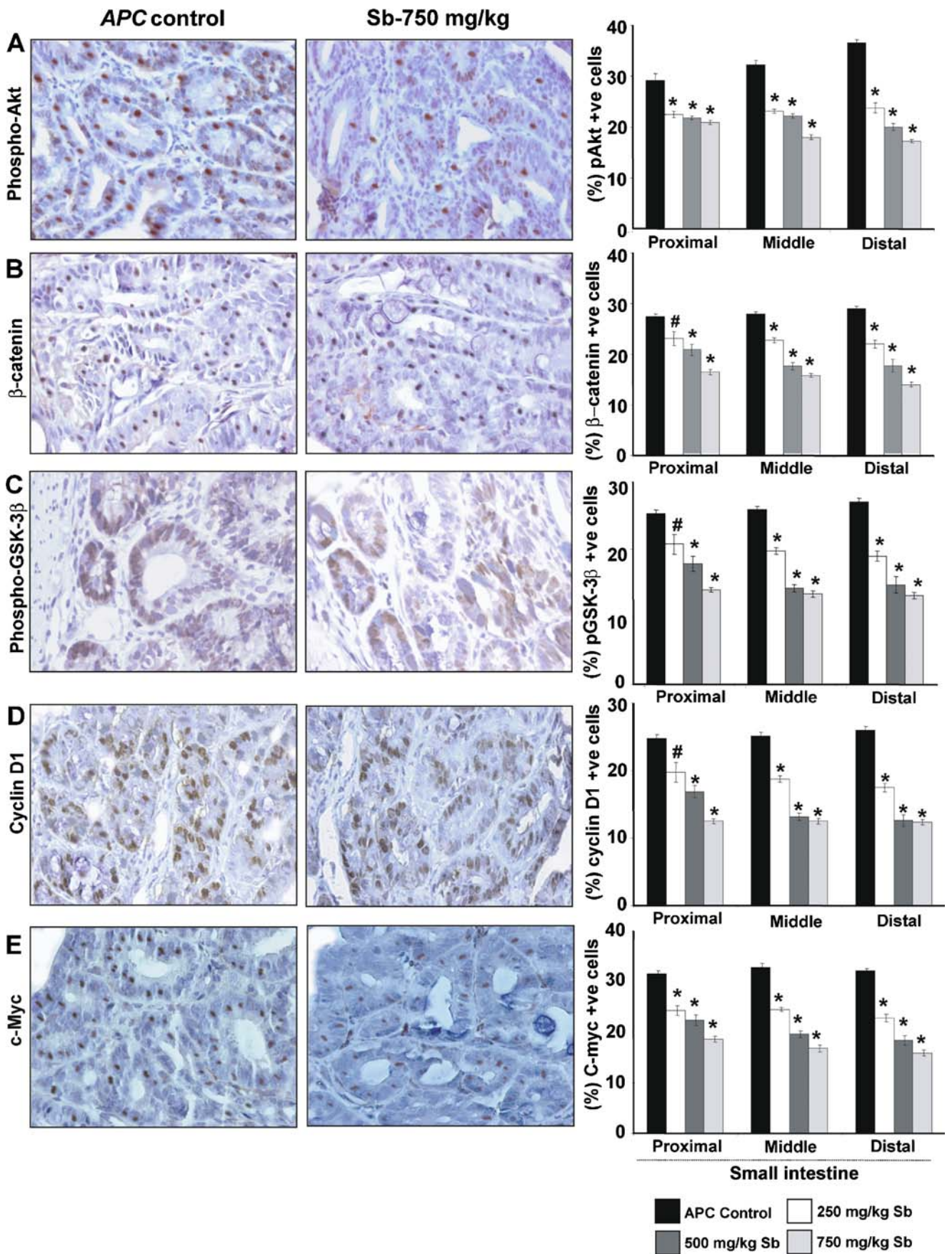
Based on our results showing a strong decrease in iNOS levels by silibinin in small intestinal polyps of *APC<sup>min/+</sup>* mice, next we analyzed its effect on iNOS activity in terms of nitrotyrosine levels in small intestinal segments (Fig. 5C) and nitrite levels in the plasma (Fig. 5D) of *APC<sup>min/+</sup>* mice. iNOS is known to produce high and sustained concentration of NO that reacts with superoxide radical to produce peroxynitrite, which in turn mediates the nitration of protein tyrosine residues to form nitrotyrosine (34). Thus, nitrotyrosine expression and nitrite levels have been used as markers for iNOS activity (34,35). Our IHC analysis showed that silibinin treatment decreases nitrotyrosine expression evidenced by low intensity of cytoplasmic staining (data not shown). Quantification of stained cells showed that silibinin at all three dose levels decreases nitrotyrosine expression in the polyps by 33–52% (*P*<0.001) in proximal, 30–52% (*P*<0.001) in middle, and 41–55% (*P*<0.001) in distal regions of small intestine (Fig. 5C). We also assessed silibinin effect on NO formation by

measuring total nitrite levels in plasma by ELISA (Fig. 5D). Silibinin decreased iNOS activity as evidenced by plasma total nitrite levels of 1.67±0.07µmol/L in control *APC<sup>min/+</sup>* mice to 0.99±0.13, 0.87±0.02 and 0.6±0.05µmol/L in 250, 500 and 750 mg/kg silibinin-treated mice, respectively. These silibinin treatments thus decreased the total/combined levels of nitrate and nitrite by 41% (*P*<0.01), 48% (*P*<0.001) and 64% (*P*<0.01), respectively (Fig. 5D). These results indicate that silibinin reduces iNOS activity mostly via the decreased expression of iNOS, further suggesting its usefulness as iNOS inhibitor in chemoprevention studies.

**DISCUSSION**

*APC<sup>min/+</sup>* mice develop numerous tumors in whole intestinal tract, primarily small intestine, and this model has genetic similarity to human CRC. The quick development of intestinal polyps also signifies the use of *APC<sup>min/+</sup>* mouse model in chemoprevention studies (6,7). Using this model, our findings demonstrate the chemopreventive efficacy of silibinin against spontaneous intestinal tumorigenesis in *APC<sup>min/+</sup>* mice. Notably, we observed that all three doses of silibinin significantly and dose-dependently decreased number and size of intestinal polyps (including both small





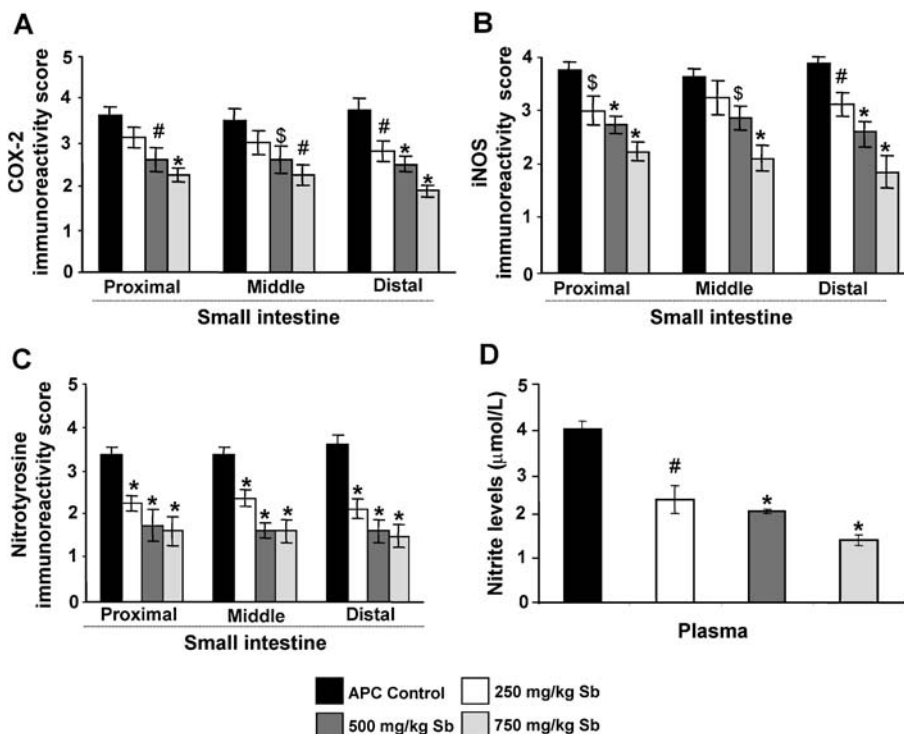
◀ **Fig. 4.** Silibinin treatment modulates aberrant  $\beta$ -catenin pathway and associated molecules selectively in small intestinal polyps of *APC<sup>min/+</sup>* mice. At the end of the study detailed in Materials and Methods, small intestinal segments were processed for IHC staining for (A) phospho-Akt, (B)  $\beta$ -catenin, (C) phospho-GSK-3 $\beta$ , (D) cyclin D1 and (E) c-Myc. The qualitative IHC (X400) and quantitative analyses of staining are shown only for small intestinal polyps, as no effect was observed for silibinin in normal crypt-villus axis. Quantitative data shown as percent positive cells represent mean $\pm$ SEM of 8 animals in each group in each case. #,  $P < 0.01$ ; \*,  $P < 0.001$  versus control; Sb, silibinin.

and large intestine), and this efficacy of silibinin was accompanied by anti-proliferative and pro-apoptotic activities. In molecular studies employing small intestinal tissues from control and silibinin-treated *APC<sup>min/+</sup>* mice, silibinin showed a significant down-regulation in Wnt/ $\beta$ -catenin pathway proteins and phospho-Akt level, together with a decrease in COX-2, iNOS and nitrotyrosine levels, and iNOS activity, selectively in the polyps. We did not observe any considerable effect of silibinin in normal crypt-villus regions of *APC<sup>min/+</sup>* or wild-type mice.

The role of hyper-proliferation and evasion of apoptosis in cancer development has been extensively studied, and increased cell proliferation and resistance to apoptosis in premalignant intestinal epithelial cells have been shown to cause the development and progression of colon tumors (36,37). Thus, identification of chemopreventive agents that

down-regulate cell proliferation and/or up-regulate apoptotic processes is recognized as useful strategy to control colon tumor growth. Our results clearly showed an *in vivo* anti-proliferative and pro-apoptotic efficacy of silibinin selectively in intestinal polyps of *APC<sup>min/+</sup>* mice together with a decrease in the number as well as size of polyps, further underscoring the overall chemopreventive effects of silibinin against CRC.

Activation of Wnt/ $\beta$ -catenin pathway in colonic epithelium is considered as one of the key events in polyp formation (27–29). Under normal conditions, APC protein binds with  $\beta$ -catenin together with axin and GSK-3 $\beta$ , thereby promoting proteolytic degradation of  $\beta$ -catenin (27). However, mutation in *APC* causes a constitutive activation of  $\beta$ -catenin pathway (28). In addition, phosphorylation of GSK-3 $\beta$  at serine 9 residue decreases its activity and also causes cytoplasmic stabilization and nuclear translocation of  $\beta$ -catenin resulting in T-cell factor-(TCF)/lymphoid enhancer factor (LEF)-dependent transcriptional activation of target genes such as cyclin D1 and c-Myc (28,30). Besides this, activation of Akt has been reported to promote the phosphorylation of GSK-3 $\beta$  that inhibits its activity, which then leads to accumulation of  $\beta$ -catenin and consequently  $\beta$ -catenin-TCF-dependent activation of more than 30 different genes including cyclin D1 and c-Myc (27,29). Both cyclin D1 and c-Myc are critical oncogenes, and their concurrent activation produces synergistic effects resulting in more aggressive cancer phenotype (38,39). It has been shown that up-regulation of  $\beta$ -catenin/TCF transcription is



**Fig. 5.** Silibinin treatment decreases COX-2, iNOS and nitrotyrosine protein levels selectively in small intestinal polyps, and nitrite levels in plasma of *APC<sup>min/+</sup>* mice. At the end of the study detailed in Materials and Methods, small intestinal segments were immunohistochemically analyzed for (A) COX-2, (B) iNOS and (C) nitrotyrosine. D, iNOS activity in plasma was estimated as described in Methods, and is expressed as  $\mu\text{mol/L}$  nitrite. The quantitative analyses of staining are shown only for small intestinal polyps, as no effect was observed for silibinin in normal crypt-villus axis. Data represent mean $\pm$ SEM of 8 animals in each group in each case. Significance was analyzed by unpaired Student's *t*-test. \$,  $P < 0.05$ ; #,  $P < 0.01$ ; \*,  $P < 0.001$  versus control; Sb, silibinin.



involved in the development of macroscopic tumors in  $APC^{min/+}$  mice (40). In the present study, high levels of  $\beta$ -catenin, phospho (ser9)-GSK-3 $\beta$ , cyclin D1 and c-Myc were observed in the small intestinal polyps of control  $APC^{min/+}$  mice, clearly showing the presence of altered and activated  $\beta$ -catenin pathway due to the functional loss of  $APC$  in these mice. Consistent with this and the inhibitory effect of silibinin on number and size of polyps in  $APC^{min/+}$  mice, we observed a selective *in vivo* down-regulation of these established molecular targets, cyclin D1 and c-Myc, in polyps of silibinin-treated mice, supporting the reduced activity of  $\beta$ -catenin pathway by silibinin treatment. Moreover, silibinin also decreased the phosphorylation of both Akt and, one of its downstream targets, GSK-3 $\beta$ , together with decreased  $\beta$ -catenin levels selectively in polyps. Overall, in  $APC^{min/+}$  mouse model, it is well-established that due to lack of functional  $APC$  protein, aberrant/uncontrolled  $\beta$ -catenin signaling is the main causative event in the development of polyps (40). Therefore, inhibition of  $\beta$ -catenin pathway would inhibit tumor initiation as well as growth in this mouse model. Thus, our results indicate that silibinin may down-regulate  $\beta$ -catenin levels either directly or indirectly through Akt-GSK-3 $\beta$  for its chemopreventive efficacy. However, further studies are needed to elucidate the mechanisms.

Several preclinical and clinical studies have shown that both iNOS and COX-2 play a central role in cancer development (33,41). Higher levels of both iNOS and NO have been reported in various cancers, including colon (34,42–44). The knocking out of iNOS gene in  $APC^{min/+}$  mice is shown to markedly reduce the number of intestinal polyps, further supporting a major role of iNOS and NO in colon carcinogenesis (43). Consistent with this report, we also observed higher levels of iNOS protein as well as its activity in polyps and elevated levels of nitrite in plasma of  $APC^{min/+}$  mice. Elevated levels of COX-2 expression have been reported in intestinal adenomas in  $APC^{min/+}$  mice (45,46). Covey *et al.* (47) have reported that pancreatic cells over-expressing COX-2 cause oxidation-mediated inactivation of tumor suppressor phosphatase and tensin homolog because of increased arachidonic acid metabolism resulting in the activation of Akt pathway. Activation of Akt as a result of increased COX-2 expression may in turn cause stabilization and activation of  $\beta$ -catenin-mediated TCF-signaling (48). In the present study, we observed a positive correlation for immunoreactivity of iNOS, nitrotyrosine and COX-2 in polyps of  $APC^{min/+}$  mice, which was decreased by silibinin treatment. However, the mechanisms of silibinin-caused decrease in iNOS and COX-2 levels in polyps, the two important molecules implicated in promoting intestinal tumorigenesis (33,45,46), remain to be established. Nevertheless, this effect of silibinin could have contributed in inhibiting the growth of polyp.

In terms of the cause-and-results relationship between the protective effect of silibinin and the decrease in the expression of beta catenin and associated molecules, we observed that silibinin treatment results in the reduction in the size and number of intestinal polyps in  $APC^{min/+}$  mice together with reduction in  $\beta$ -catenin and a resultant decrease in the expression of cyclin D1 and c-Myc, the downstream targets of  $\beta$ -catenin pathway. Since cyclin D1 and c-Myc are oncogenes associated with increased proliferation of

cancerous cells, it seems reasonable to assume that a decrease in  $\beta$ -catenin levels by silibinin might be causally responsible for the observed reduction in the number and size of the polyps in the intestinal tract of  $APC^{min/+}$  mice. With regard to upstream of  $\beta$ -catenin, we found that silibinin also inhibits the phosphorylation of Akt, which might be responsible for the activation of GSK-3 $\beta$  in terms of reduced phosphorylation at Ser 9 residue by silibinin (activation of Akt promotes the phosphorylation of GSK-3 $\beta$  at Ser 9 inhibiting its activity), which then promotes  $\beta$ -catenin degradation. Thus, silibinin targeting  $\beta$ -catenin pathway through Akt and GSK-3 $\beta$  by decreasing nuclear  $\beta$ -catenin levels together with down-regulation of cyclin D1 and c-Myc could be one of the possible underlying cause-and-effect mechanisms of its chemopreventive potential against intestinal polyps in  $APC^{min/+}$  mice as observed in the present study. In summary, we observed that silibinin treatment inhibits multiplicity as well as growth of intestinal polyps in  $APC^{min/+}$  mice, without any apparent adverse health effects, by decreasing cell proliferation, inducing apoptosis, and modulating  $\beta$ -catenin pathway, COX-2 and iNOS expression. Since almost 90% of FAP patients develop small intestinal polyps together with colonic polyps that remain a significant risk factor following colectomy (2,6), findings of the present study have a translational potential in offering a relatively non-toxic alternative for the prevention of intestinal polyps in FAP patients as well as the reduction of associated morbidity and mortality.

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