

## Farnesol inhibits cell proliferation and induces apoptosis after partial hepatectomy in rats<sup>1</sup>

Farnesol inibe a proliferação celular e induz a apoptose após a hepatectomia parcial em ratos

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### ABSTRACT

**Purpose:** To study farnesol (FOH) effects on liver regeneration after 70% partial hepatectomy (PH) in rats. **Methods:** Animals received FOH (25 mg/100 g body weight/day) or corn oil (CO, 0.25 mL/100 g body weight/day, controls). After a 2 week-treatment, all animals were subjected to PH and euthanized at different time points (0 h, 0.5 h, 4 h, 8 h, 18 h and 24 h) after surgery. Hepatic cell proliferation (PCNA positive nuclei) and apoptosis (fluorescence microscopy) were evaluated. **Results:** Compared to CO treatment, FOH treatment inhibited ( $p < 0.05$ ) cell proliferation at 24h (S phase of the cell cycle) after PH. This was preceded by an induction of apoptosis 0.5 h ( $p < 0.05$ ;  $G_0/G_1$  transition phase) after surgery. **Conclusion:** The results of the present study suggest that apoptosis induction could be associated with the reduced number of cells at the S phase observed in FOH group. These novel *in vivo* data reinforce FOH as a promising chemopreventive and therapeutic agent against cancer.

**Key words:** Farnesol. Hepatectomy. Cell Proliferation. Apoptosis. Rats.

### RESUMO

**Objetivo:** Estudar o efeito do farnesol (FOH) durante a regeneração hepática em ratos submetidos à Hepatectomia Parcial (HP) a 70%. **Métodos:** Os animais foram tratados com FOH (25 mg/100g de peso corpóreo/dia) ou óleo de milho (OM, 0,25 mL/100g de peso corpóreo/dia, grupo controle). Depois de 2 semanas de tratamento, todos os animais foram submetidos à HP e eutanaziados em diferentes momentos (0h, 30min., 4h, 8h, 18h, 24h.) após o procedimento cirúrgico. Foi avaliada a proliferação celular (imunohistoquímica para PCNA) e a apoptose (microscopia de fluorescência). **Resultados:** Em comparação aos animais controles, animais tratados com FOH apresentaram menor ( $p < 0,05$ ) proliferação celular 24h. (fase S do ciclo celular) após a HP. Tal efeito foi precedido de uma indução de apoptose 30min. ( $p < 0,05$ ; transição entre as fases  $G_0/G_1$  do ciclo celular) após a cirurgia. **Conclusão:** Os resultados do presente estudo sugerem que a indução da apoptose pode estar associada com o menor número de células na fase S observadas nos animais tratados com FOH. Essa nova evidência *in vivo* reforça o farnesol como um promissor agente preventivo e terapêutico contra o câncer.

**Descritores:** Farnesol. Hepatectomia. Proliferação de Células. Apoptose. Ratos.

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### Introduction

Cancer is an important global public health problem. The American Cancer Society projected 1,437,180 new cancer cases and 565,650 deaths from cancer in 2008<sup>1</sup>. Despite advances in therapy, prognosis for patients with cancer remains poor. Thus chemoprevention is considered a relevant strategy for cancer control<sup>2</sup>.

Diet-derived isoprenoids present inhibitory effect against colon<sup>3,4</sup>, pancreas<sup>5,6</sup> and liver<sup>7,8</sup> carcinogenesis. They comprise a class of substances with over 20,000 constituents widely distributed in fruits and vegetables. Farnesol (FOH), a 15-carbon isoprenoid present in orange peel and lemon-grass oil

and strawberries, has been considered a promising cancer chemopreventive<sup>8,9</sup> and therapeutic agent<sup>10</sup>. Although FOH inhibited cell proliferation and induced apoptosis *in vitro*, *in vivo* information is scarce<sup>11</sup>. In rodents, these actions were observed during chemically induced pancreas<sup>5,6</sup> and liver<sup>8</sup> carcinogenesis, but not in models specifically designed for the study of cell proliferation.

The 70% partial hepatectomy (PH) is considered a classic, highly regulated and orchestrated model for the study of cell proliferation during liver regeneration<sup>12</sup>. After PH, most remaining hepatocytes promptly enter and progress in the cell cycle in a synchronous manner. The first stage (priming), in which hepatocytes undergo transition from resting state ( $G_0$ ) to one in

which they become capable to proliferate ( $G_1$ ), lasts 4-6 h after PH<sup>14</sup>. Then, these primed hepatocytes enter a progression stage, with DNA synthesis starting around 18 h and peaking at 24 h after PH<sup>12-14</sup>. The objective of the present study was to investigate the effect of FOH administration in rats submitted to PH, a synchronized and well characterized model for the study of cell proliferation.

## Methods

### Chemicals

FOH (*trans, trans*-3, 7, 11-trimethyl-2, 6, 10-dodecatrien-1-ol; 96%) was purchased from Aldrich. 3,3'-diaminobenzidine and bovine serum albumin were purchased from Sigma. The commercial diet was purchased from Purina®. Corn oil (CO) was Mazola®. Polyclonal anti-proliferative cell nuclear antigen (PCNA) rat antibody, secondary biotinylated antibody and streptavidin-biotin-peroxidase complex were purchased from Dako.

### Animals and treatment

Male Wistar rats from the colony of the Faculty of Pharmaceutical Sciences, initially weighing 50 g maintained in cages with four animals, at constant temperature (22°C), with 12 h light-dark cycle and receiving water and commercial diet *ad libitum*, were used.

Figure 1 illustrates the experimental design. At the end of a 7-day acclimatization period, 102 animals were randomly divided into 2 experimental groups. FOH group received FOH (25 mg/100 g bw) dissolved in CO (0.25 mL/100 g bw). CO group (control) received only CO. Both treatments were performed by gavage daily for 2 consecutive weeks. Afterwards, all animals were submitted to a 70% PH as previously described<sup>15</sup> and euthanized at 0 h, 0.5 h, 4 h, 8 h, 18 h and 24 h after surgery. The last dose of FOH was administrated 2 h before euthanasia. The study was carried out within the guidelines of the Brazilian College of Animal Experiments (COBEA) and was approved by the Faculty of Pharmaceutical Sciences Ethic Committee for the Care and Use of Laboratory Animals (protocol number 16).

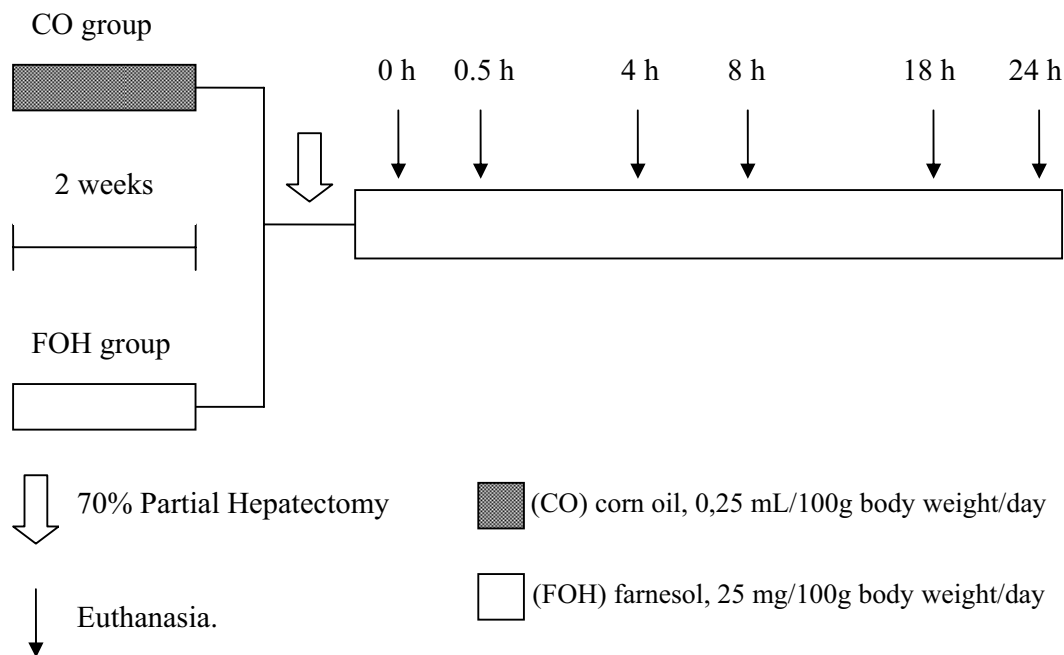


FIGURE 1 - Experimental design

### Hepatic cell proliferation analysis

To evaluate cell proliferation, representative fragments of each liver lobe were fixed in methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for 24 h and included in paraffin. Histological sections of 5  $\mu$ m were processed in order to detect PCNA-positive hepatocytes as described by Fonseca *et al.*<sup>16</sup>. Basically, after paraffin removal, endogenous peroxidase was blocked by 20% hydrogen peroxide in methanol for 0.5 h. Thereafter, the sections were incubated overnight at 4°C with

primary anti-PCNA rat antibody at a 1:1600 dilution in 1% bovine serum albumin. Finally, the sections were incubated for 1 h with secondary biotinylated antibody and thereafter the streptavidin-biotin-peroxidase complex was applied. Peroxidase binding sites were detected by incubation with 3,3'-diaminobenzidine (0.5%) and hydrogen peroxide (0.1%) dissolved in phosphate buffer saline (PBS), for 2 min at room temperature. Sections were counterstained with hematoxylin. The entire liver section was

analyzed using light microscope. Cell proliferation index (CPI) was expressed as the number of PCNA-positive nuclei/mm<sup>2</sup> histological liver section<sup>8</sup>.

#### Hepatic apoptosis evaluation

Hepatic apoptotic bodies (AB) were quantified by fluorescence microscopy as previously described<sup>8</sup> using a Nikon microscope equipped with an epifluorescence unit. This method is based on the strong eosin fluorescence of AB in hematoxylin and eosin (H&E) stained liver tissues submitted to blue light (450-490 nm). Identification of AB was confirmed by switching the microscope system from blue to transmitted light and using morphological criteria established by Goldsworth *et al.*<sup>17</sup>. AB were represented by acidophilic bodies with fragmentation or lack of chromatin accompanied by cytoplasmic condensation and/or fragmentation<sup>17</sup>. If single cells or clusters of directly neighbouring cell contained multiple AB, these were assumed to be derived from the same apoptotic cell and were counted as only one event. The entire liver section was analyzed. Apoptotic index (AI) was expressed as the number of hepatic AB/mm<sup>2</sup> of histological section<sup>8</sup>.

#### Statistical analysis

Sigma Stat 2.0 program was used for statistical analysis. For all analyzed parameters a two-way ANOVA followed by a Tukey test was performed in order to identify any significant differences between CO and FOH group in all euthanasia time points. In all cases a level of significance of  $p < 0.05$  was applied.

#### Results

##### Absolute and relative liver weights

Absolute and relative liver weights of rats treated with FOH or CO and submitted to 70% PH are shown in Table 1. No statistically significant ( $p > 0.05$ ) differences were observed between experimental groups in all euthanasia time-points regarding absolute and relative liver weights. These results suggest that FOH did not present toxicity. FOH dosage was based on our previous study in which it did not present any apparent toxicity when administered during 8 consecutive weeks to rats submitted to a hepatocarcinogenesis model<sup>8</sup>.

**TABLE 1** - Absolute and relative liver weight of rats treated for two consecutive weeks with CO or FOH and submitted to PH

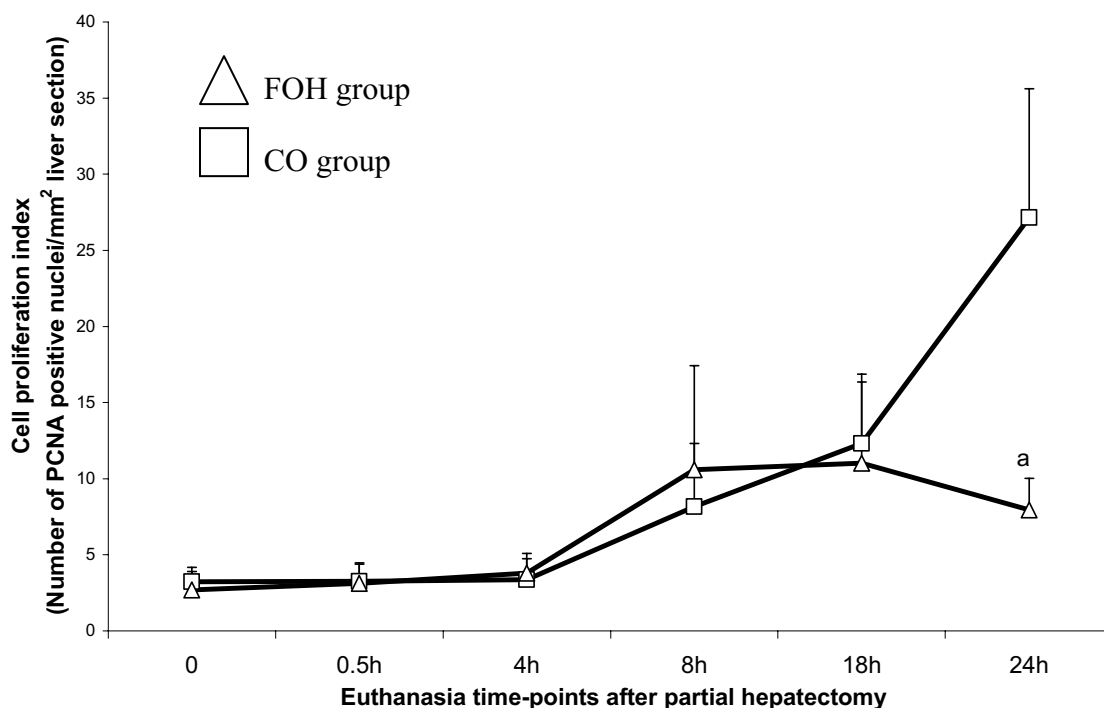
Absolute liver weight (g)						
Euthanasia	0h (n=9)	0.5h (n=8)	4h (n=9)	8h (n=8)	18h (n=8)	24h (n=9)
time points						
CO	3,66 ± 0,2	3,45 ± 0,1	3,34 ± 0,2	3,75 ± 0,1	4,11 ± 0,1	4,29 ± 0,2
FOH	3,72 ± 0,2	3,42 ± 0,2	3,64 ± 0,1	3,94 ± 0,1	4,38 ± 0,2	4,38 ± 0,2
Relative liver weight (g/100g of body weight)						
Euthanasia	0h (n=9)	0.5h (n=8)	4h (n=9)	8h (n=8)	18h (n=8)	24h (n=9)
time points						
CO	1,19 ± 0,1	1,15 ± 0,1	1,1 ± 0,1	1,22 ± 0,0	1,41 ± 0,1	1,45 ± 0,1
FOH	1,18 ± 0,1	1,15 ± 0,1	1,19 ± 0,0	1,32 ± 0,1	1,46 ± 0,0	1,47 ± 0,1

\* Data are means ± SEM.

#### FOH effects on hepatic cell proliferation and apoptosis

Figure 2 shows CPI of animals of FOH and CO groups. Until 18 h after surgery no statistically significant differences ( $p > 0.05$ ) were observed regarding FOH and CO groups CPI.

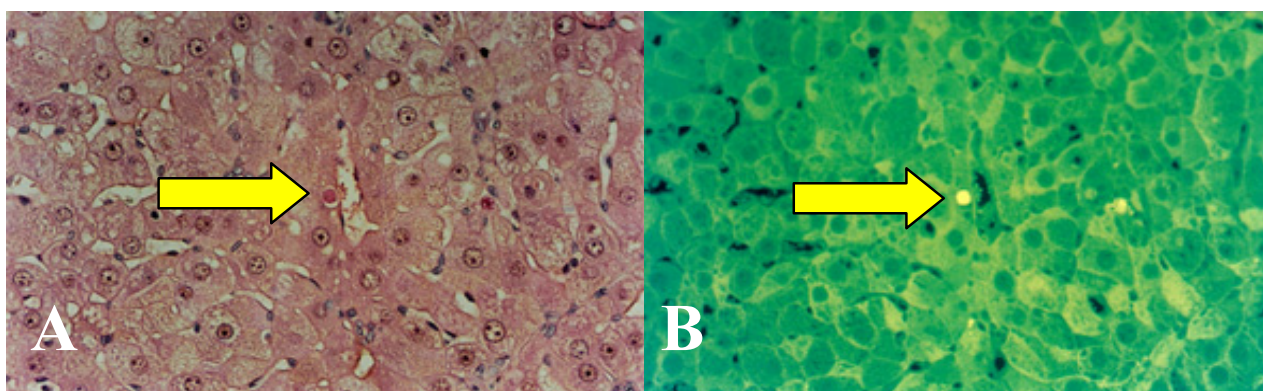
In CO group, the highest CPI was observed 24 h after surgery. Compared to CO group, FOH group presented smaller ( $p < 0.05$ ) CPI 24 h after PH.



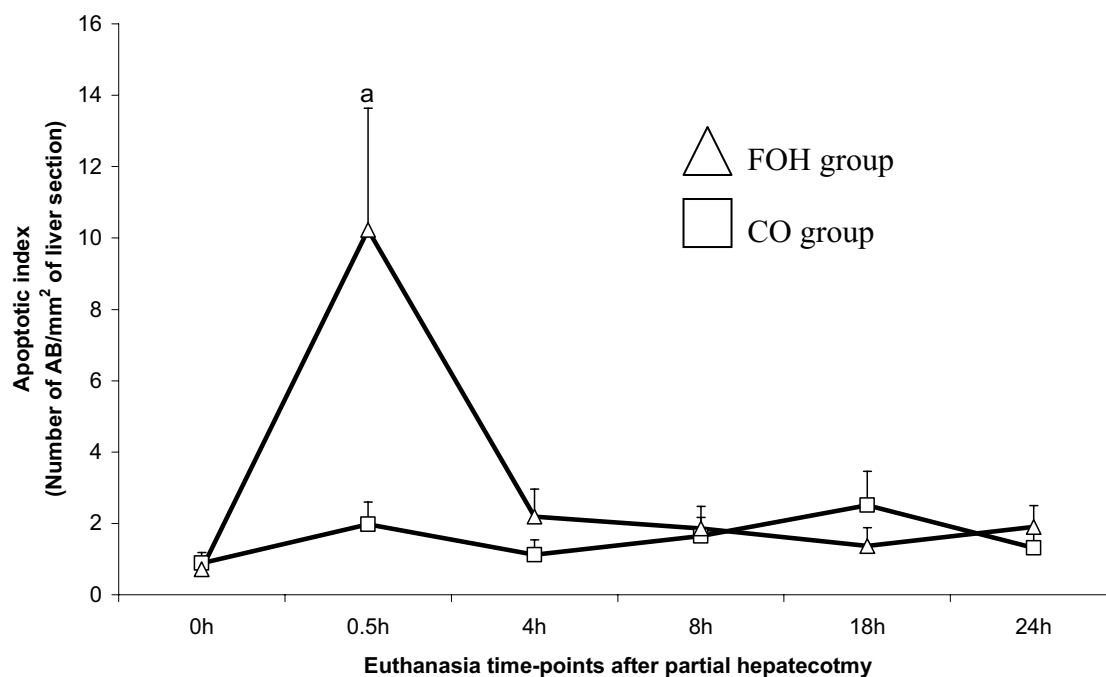
**FIGURE 2** - Hepatic cell proliferation index (CPI) of rats treated for 2 consecutive weeks with CO or FOH and submitted to PH. In both CO and FOH treatment, the number of animals analyzed varied from 8 to 9 in each euthanasia time-point. Data are means  $\pm$  SEM. Statistics by two-way ANOVA followed by Tukey test: a) statistically different from CO group in the same euthanasia time-point

In the present study apoptosis was evaluated by counting AB using fluorescence microscopy<sup>8</sup>. Advantages of this method are the fast identification of AB due to their strong fluorescence in H&E-stained liver sections and increased sensitivity, since small fluorescent AB, usually not recognized by transmitted light microscopy, can also be identified<sup>8</sup>. Figure 3 shows an example of

a fluorescent hepatic AB stained with H&E. Figure 4 shows AI of animals of FOH and CO groups. Compared to CO group, FOH group presented higher ( $p < 0.05$ ) AI 0.5 h after PH. No differences ( $p > 0.05$ ) were observed between FOH and CO groups in the other euthanasia time-points.



**FIGURE 3** - Hepatic apoptotic body visualized by transmitted light microscopy (A) and fluorescent microscopy (B). 40X objective



**FIGURE 4** - Hepatic apoptosis index (AI) of rats treated with CO or FOH for 2 consecutive weeks and submitted to PH. In both CO and FOH treatment, the number of animals analyzed varied from 8 to 9 in each euthanasia time-point. Data are means  $\pm$  SEM. Statistics by two-way ANOVA followed by Tukey test: a) statistically different from CO group in the same euthanasia time-point

## Discussion

The effects of chemopreventive agents on cell proliferation and apoptosis can be investigated in detail using 70% PH, a classic and synchronous model of liver regeneration<sup>12</sup>. Since hepatic regeneration in rodents is similar to that observed in humans, the results obtained from rodents can be also applicable to the human liver<sup>18</sup>. The priming stage ( $G_0/G_1$  transition) comprises the first 4-6 h, followed by progression that lasts 12-16 h. Hepatic DNA synthesis (S phase) starts around 18 h and peaks at 24 h after surgery<sup>12-14</sup>.

Cell proliferation has been suggested to involve a regulated passage through some checkpoints which ensure the proper timing of cell cycle events<sup>19</sup>. Compounds that prevent cell cycle progression can be used as negative regulators of cell proliferation in proliferative diseases such as cancer<sup>20</sup>. FOH induced  $G_0/G_1$  arrest which led to apoptosis and inhibition of cell proliferation in lung and leukemia cells<sup>21,22</sup>. In the present study, rats treated with FOH presented a reduced number of PCNA positive nuclei 24 hours (S phase) after 70% PH. In addition, FOH effect on cell proliferation was preceded by apoptosis induction started 0.5 h ( $G_0/G_1$  transition) after surgical procedure.

Apoptosis is considered an ideal way of eliminating undesired or genetically altered cells without the induction of inflammatory response<sup>22</sup>. Arsenite<sup>23</sup>, manganese<sup>24</sup> and 5-(N,N-hexamethylene)-amiloride<sup>25</sup>, when administered subcutaneously after PH, induced apoptosis 4h after surgery. Thus considering the present and previously data<sup>23-25</sup> it seems that apoptosis occurs preferentially during the priming stage of liver regeneration. The

differences regarding the time of apoptosis induction between these above mentioned studies and the present study could be related to the fact that FOH administration by gavage was started 2 weeks before the PH which could increase the hepatic concentration of this isoprenoid.

We suggest that similarly to *in vitro* studies, in the present study FOH induced a  $G_0/G_1$  cell cycle arrest that led to apoptosis induction after PH. This could be associated to the reduced number of PCNA positive cells at the S phase as also described for retinoic acid<sup>14</sup>, quercetin<sup>26</sup> and 5-(N,N-hexamethylene)-amiloride<sup>25</sup>. In those studies rats submitted to PH and treated with these substances presented inhibition of DNA synthesis that was also preceded by apoptosis induction starting during early  $G_1$  phase.

In summary, the results of the present study reinforce FOH as an attractive candidate for cancer prevention<sup>8,9</sup> and therapy<sup>10</sup>. In order to provide further information regarding FOH actions on cell cycle phases, future studies could focus on the molecular pathways involved in both apoptosis and cell proliferation *in vivo*.

## Conclusion

The farnesol induces apoptosis and decreases cell proliferation during liver regeneration after partial hepatectomy.

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#### Acknowledgement

The authors would like to thanks Miss Sylvania M.P. Neves for providing the care and maintenance of the animals.

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Conflict of interest: none  
Financial source: FAPESP, CNPq and CAPES

Received: March 18, 2009  
Review: May 12, 2009  
Accepted: June 16, 2009

#### How to cite this article

Chagas CEA, Vieira A, Ong TP, Moreno FS. Farnesol inhibits cell proliferation and induces apoptosis after partial hepatectomy in rats. *Acta Cir Bras*. [serial on the Internet] 2009 Sept-Oct;24(5). Available from URL: <http://www.scielo.br/achb>

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