Halofuginone may suppresses azoxymethane-induced serum tumor necrosis factor-a synthesis and aberrant crypt foci progression in rat colon

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ABSTRACT

The aim of this research was to investigate the effect of halofuginone on the progression of azoxymethane-induced colon cancer in rats. A total of 38 male Wistar albino rats were divided into 4 groups: Control (n=8), Halofuginone (n=10, 0.4 mg/kg, PO, SID), Cancer (n=10, azoxymethane, 15 mg/kg, IP, once a week for two weeks) and Cancer + Halofuginone (n=10). After 18 weeks, blood samples were taken under anesthesia and all animals were sacrificed. Aberrant crypt foci in the colon were stained with methylene blue. Blood cytokines, thiobarbituric acid reactive substances, 13,14-dihydro-15-keto-prostaglandin F2α levels, hemogram and biochemical values were measured. The tumor necrosis factor-a level in the Cancer group was higher (P<0.05) than in other groups, while higher numbers of aberrant crypt foci were found in the Cancer group compared with the Cancer + Halofuginone group (P<0.05). In summary, it may be stated that halofuginone may warrant evaluation as a supportive drug in the treatment of colon cancer in the future.

Key words: Aberrant crypt foci, Colon cancer, Cytokines, Halofuginone.

INTRODUCTION

Halofuginone (HLF) has been used for a long time in the treatment of coccidiosis and cryptosporidiosis in veterinary medicine. Since the 1990’s, HLF has been widely investigated in human medicine, and it has been reported that HLF has specific inhibitory effects on collagen type 1 synthesis (Pines et al., 2000). Beneficial effects of HLF have been reported in some cancer types (Gavish et al., 2002; Abramovitch et al., 2004). Colorectal cancer is the third most common malignancy in the world, and is the fourth most common cause of death from cancer. Colon cancer is diagnosed in over one million people each year worldwide (Landskron et al., 2014).

Aberrant crypt foci (ACF) are accepted as the earliest neoplastic lesion of colon cancer, and it is believed that ACF may be a precursor to colon cancer. ACF are used as a biomarker of colon cancer in humans and rats. The size and number of ACF increase as the cancer progresses, so they provide information about the stage of the cancer. There is a positive correlation between the incidence of ACF and cancer severity (Alrawi et al., 2006; Gupta and Schoen, 2008; Wargovich et al., 2010). Treatment outcomes may be evaluated according to the number, size and multicrypt features of ACF (Raju, 2008).

Cytokines, low-molecular-weight proteins, play roles in communication among cells and cancer progression. Cytokines regulate proliferation, differentiation, migration, invasion, survival and death of cancer cells (Lee and Margolin, 2011; Landskron et al., 2014). Tumor necrosis factor (TNF)-a (Wang and Lin, 2008), interleukin (IL)-2 (Baier et al., 2005), IL-6 (Landskron et al., 2014), IL-10 (Lin and Karin, 2007) and 13,14-dihydro-15-keto-prostaglandin F2α (PGM) (Dunzendorfer et al., 1981) may play role in the cancer progress. In addition, reactive oxygen species (ROS) also play a role in cancer development, as well. After lipid peroxidation, thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) are produced, and their levels are accepted as markers of lipid peroxidation (Mayne, 2003; Landskron et al., 2014).

The aim of this research was to determine the effects of HLF on TNF-a, IL-2, IL-6 and IL-10 levels, ACF formation, PGM and TBARS levels in azoxymethane-induced colon cancer in rats, and to find a new therapeutic approach to the treatment of colon cancer.

MATERIALS AND METHODS

A total of 38 male Wistar albino rats (297.70 ± 4.90 g, 12–16 weeks old) were used and research procedure was approved by SUDAM (No: 2014/18). The rats were housed at a continuous temperature (25°C) and humidity (55%) at 8:00–20:00 h light and 20:00–8:00 h dark cycle. The animals were fed with standard water and food ad libitum. Rats were divided into 4 groups; 1-CNT group (Control group, n=8): Normal sterile saline solution was administered (SID) by gastric lavage during 18 weeks, 2-HLF group (Halofuginone group, n=10, 0.4 mg/kg, PO, SID), Cancer (n=10, azoxymethane, 15 mg/kg, IP, once a week for two weeks) and Cancer + Halofuginone (n=10). After 18 weeks, blood samples were taken under anesthesia and all animals were sacrificed. Aberrant crypt foci in the colon were stained with methylene blue. Blood cytokines, thiobarbituric acid reactive substances, 13,14-dihydro-15-keto-prostaglandin F2α levels, hemogram and biochemical values were measured. The tumor necrosis factor-a level in the Cancer group was higher (P<0.05) than in other groups, while higher numbers of aberrant crypt foci were found in the Cancer group compared with the Cancer + Halofuginone group (P<0.05). In summary, it may be stated that halofuginone may warrant evaluation as a supportive drug in the treatment of colon cancer in the future.

Key words: Aberrant crypt foci, Colon cancer, Cytokines, Halofuginone.
RESULTS AND DISCUSSION

During the study period, 1, 2, 3 and 2 rats died in the CNT, HLF, CAN and CAN+HLF groups, respectively. Final body weights of rats were 513.57±44.35; 518.57±19.53, 438.28±23.41 and 466.71±20.80 g in CNT, HLF, CAN and CAN+HLF groups, respectively. There was no statistically significant difference for body weight (P>0.05).

ACF was not observed in the rats both CNT and HLF groups. However, all azoxymethane administered rats developed ACF in CAN and CAN+HLF groups. Higher ACF score was found in the CAN group compared with the CAN+HLF group (P<0.05, Figure 1, Table 1). In the macroscopic examination, although there were no lesions in the CNT or HLF groups, macroscopic lesions (0.3-0.5 mm) were observed in the CAN (4/7) and CAN + HLF (2/7) groups (Fig 2 and 3).

Serum TNF-α, IL-2, IL-6, IL-10 and TBARS, and plasma PGM levels are presented in Table 1. The statistically significant (P<0.05) higher TNF-α level was measured in the CAN group when compared other groups (P>0.05).

Hemogram and serum biochemical parameters are shown in Table 2. Lower hemoglobin level in the CAN+HLF group, and higher cholesterol and triglyceride levels in the HLF group were determined (P<0.05).

Colon cancer is the most frequently observed lethal cancer type in the world (Landskron et al., 2014). In the literature, the effect of HLF on colon cancer has been examined in the nude mouse (Chen et al., 2015), but its effects on TNF-α, IL-2, IL-6 and IL-10 levels, ACF score, the main marker of colon cancer, PGM and TBARS levels have not been investigated.

In this study, ACF score and macroscopic lesions were not seen in the CNT or HLF groups. The ACF score in the CAN group was significantly higher (P<0.05) than in the CAN + HLF group (Fig 1, Table 1). In addition, macroscopic lesions were observed in the CAN (4/7) and in the CAN + HLF (2/7) groups. ACF are early precancerous lesions of the colon and ACF are accepted as a biomarker of colon cancer. There is a positive correlation between the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CNT</th>
<th>HLF</th>
<th>CAN</th>
<th>CAN+HLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACF (score)</td>
<td>0(^a)</td>
<td>0(^a)</td>
<td>3(^b)</td>
<td>2(^b)</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>113.32 ± 6.06(^b)</td>
<td>147.61 ± 23.00(^a)</td>
<td>214.28 ± 36.95(^a)</td>
<td>129.28 ± 16.77(^a)</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>157.38 ± 4.97</td>
<td>154.69 ± 2.40</td>
<td>170.74 ± 9.85</td>
<td>193.10 ± 36.27</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>25.02 ± 2.11</td>
<td>29.54 ± 5.03</td>
<td>35.26 ± 5.89</td>
<td>32.40 ± 5.47</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>215.39 ± 13.47</td>
<td>212.53 ± 8.46</td>
<td>223.96 ± 19.54</td>
<td>220.03 ± 11.38</td>
</tr>
<tr>
<td>PGM (pg/mL)</td>
<td>219.66 ± 37.91</td>
<td>250.69 ± 33.52</td>
<td>252.56 ± 27.99</td>
<td>300.05 ± 29.41</td>
</tr>
<tr>
<td>TBARS (μM)</td>
<td>4.63 ± 0.59</td>
<td>4.26 ± 0.67</td>
<td>3.45 ± 0.33</td>
<td>3.45 ± 0.37</td>
</tr>
</tbody>
</table>

CNT: control group, HLF: halofuginone group, CAN: cancer group, CAN + HLF: cancer + halofuginone group, ACF: aberrant crypt foci, TNF-α: tumor necrosis factor-α, IL-2; interleukin 2, IL-6; interleukin 6, IL-10; interleukin 10, PGM: 13,14-dihydro-15-keto-prostaglandin F2α, TBARS: thiobarbituric acid reactive substances. \(^a\), \(^b\), \(^c\): Different letters in the same row indicate that differences are statistically significant (P<0.05).
size and number of ACF and the severity of colon cancer (Alrawi et al., 2006; Gupta and Schoen, 2009; Wargovich et al., 2010). It has been reported that HLF may induce apoptosis and reduce cancer volume in experimentally induced colon cancer in the nude mouse (Chen et al., 2015). It may also have antiangiogenic, antimetastatic and antiproliferative effects, increase apoptosis/necrosis, and decrease collagen content in other cancer types (Gavish et al., 2002; Grudzien et al., 2010; Jordan and Zeplin, 2012).

Further, HLF may enhance the effects of chemotherapeutic agents on cancer (Leiba et al., 2012). It has been reported that the beneficial effects of some agents used in colon cancer may depend on their antioxidant, antiinflammatory, antiproliferative and antiangiogenic effects, as well as their

**Table 2: Effect of halofuginone on hemogram and biochemical values in experimentally induced colon cancer (mean ± SE).**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CNT</th>
<th>HLF</th>
<th>CAN</th>
<th>CAN + HLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (×10^3/ìL)</td>
<td>6.81 ± 0.61</td>
<td>7.17 ± 0.72</td>
<td>8.34 ± 0.68</td>
<td>9.61 ± 1.07</td>
</tr>
<tr>
<td>RBC (×10^6/ìL)</td>
<td>8.95 ± 0.14</td>
<td>8.93 ± 0.09</td>
<td>8.65 ± 0.46</td>
<td>8.92 ± 0.40</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>14.88 ± 0.27^a</td>
<td>14.58 ± 0.23^b</td>
<td>13.98 ± 0.76^ab</td>
<td>12.85 ± 0.51^b</td>
</tr>
<tr>
<td>HTC (%)</td>
<td>45.64 ± 0.65</td>
<td>44.65 ± 0.07</td>
<td>44.28 ± 2.42</td>
<td>40.48 ± 1.34</td>
</tr>
<tr>
<td>PLT (×10^9/ìL)</td>
<td>505.71 ± 14.39</td>
<td>485.42 ± 22.26</td>
<td>509.28 ± 27.10</td>
<td>456.71 ± 71.07</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>176.14 ± 34.63</td>
<td>155.42 ± 31.63</td>
<td>247.42 ± 27.64</td>
<td>200.57 ± 31.28</td>
</tr>
<tr>
<td>TB (mg/dL)</td>
<td>0.02 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>86.00 ± 7.42</td>
<td>80.00 ± 6.68</td>
<td>97.57 ± 23.26</td>
<td>79.00 ± 14.01</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>281.71 ± 47.87</td>
<td>300.42 ± 48.61</td>
<td>323.85 ± 64.65</td>
<td>310.71 ± 53.69</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>0.57 ± 0.20</td>
<td>0.57 ± 0.20</td>
<td>3.00 ± 2.00</td>
<td>1.57 ± 0.42</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>4.26 ± 0.14</td>
<td>4.26 ± 0.74</td>
<td>4.17 ± 0.22</td>
<td>4.02 ± 0.12</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>4.38 ± 0.10</td>
<td>4.00 ± 0.17</td>
<td>4.29 ± 0.09</td>
<td>4.61 ± 0.24</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.46 ± 0.03</td>
<td>0.48 ± 0.04</td>
<td>0.49 ± 0.01</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>CHL (mg/dL)</td>
<td>65.42 ± 2.92^ab</td>
<td>77.85 ± 12.49^a</td>
<td>53.28 ± 4.80^ab</td>
<td>47.47 ± 3.92^a</td>
</tr>
<tr>
<td>TRG (mg/dL)</td>
<td>104.00 ± 14.63^a</td>
<td>129.00 ± 27.15^a</td>
<td>57.28 ± 6.87^b</td>
<td>55.28 ± 7.95^b</td>
</tr>
</tbody>
</table>

**Fig 1:** Methylene blue stain of colon tissue. A: Control group, B: Halofuginone group, C and E: Cancer group, aberrant crypt foci (arrows, score: 4), D and F: Cancer + Halofuginone group, aberrant crypt foci (arrows, score: 2), (A–D: ×40, E–F: ×100).

**Fig 2:** Tumor mass in colon (arrow), A: Cancer group, B: Cancer + Halofuginone group.
ability to induce apoptosis (Khan et al., 2013; Afrin et al., 2016). In this study, the antiproliferative and antiangiogenic effects of HLF, combined with induction of apoptosis may be responsible for the decrease in size and number of ACF.

In the current research, the TNF-a level in the CAN group was higher (P<0.05) than all other groups (Table 1). TNF-a levels increase in colon cancer (Wang and Lin, 2008), and TNF-a promotes cancer progression via induction of nitric oxide and reactive oxygen species production (Lin and Karin, 2007; Landskron et al., 2014). In addition, levels of IL-6 and TNF-a may simultaneously increase with ACF levels (Muthu et al., 2015; Kangwan et al., 2016). In the present research, HLF inhibited the increase of TNF-a level in CAN+HLF group. It has been reported that HLF may inhibit NF-kB activation (Leiba et al., 2006). In this current research, HLF may decrease TNF-a level by inhibition of NF-kB, which is a transcription factor that plays a key role in cytokine synthesis (Hayden and Ghosh, 2014). In this study, there were no statistically significant changes in IL-2, IL-6 or IL-10 levels in any of the groups (Table 1). IL-2 may have an antitumor effect (Arenas-Ramirez et al., 2015) and levels of IL-2 decrease in colon cancer (Baier et al., 2005). However, HLF may increase IL-2 levels (Jin et al., 2014). It has been reported that levels of IL-6 may increase in colon cancer (Landskron et al., 2014). HLF may decrease IL-6 levels (Liang et al., 2013) and an antagonist of IL-6 may be beneficial in the treatment of colon cancer. IL-10 plays a role in the inhibition of angiogenesis and regulation of apoptosis during tumor progression (Lin and Karin, 2007; Landskron et al., 2014), and HLF decreases IL-10 levels (Jin et al., 2014). In this research, the lack of effect of HLF on IL-2, IL-6 and IL-10 levels may be a consequence of the model, animals, sampling time, severity and period of the colon cancer, duration of the experiment or, especially, the dose of HLF. However, the inhibitory effect of HLF on TNF-a is important and HLF may have a valuable effect on the progression of colon cancer. In this research, PGM and TBARS levels were unchanged in all groups (P>0.05, Table 1). These results may be derived from sufficient antioxidant capacity of rats.

In conclusion, HLF may have beneficial effects in the therapy and/or prophylaxis of colon cancer, since it inhibits TNF-a synthesis and ACF progression. Further research is required to explore the combination of HLF with other chemotherapeutics in cancer treatment.

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REFERENCES


