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Research Article

Antiulcerogenic Effects of Kolaviron: Inhibition of Apoptosis induced by Indomethacin in the intact Rat Stomach and Rat Gastric Mucosa Cell line

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ABSTRACT: The crude extracts of the seed of *Garcinia kola* (GK) and its constituent biflavonoid, Kolaviron (KV), have been shown to effectively protect the stomach of rats from ulceration induced by HCl/Ethanol mixture as well as indomethacin. However, the anti-ulcerogenic mechanisms are yet to be fully elucidated. Since apoptosis has been identified as a potent factor in ulcerogenesis, the study was designed to examine the anti-apoptotic effect of KV in intact and cultured gastric cells (RGM-1) of rat. Confluent Rat Gastric Mucosal (RGM-1) cells were challenged with medium containing 500uM indomethacin in the presence or absence of 0.5ug/ml and 5.0ug/ml KV. Cell viability was measured by the MTT [3-(4, 5- dimethylthiazol-2-yl)-2,5-deiphenyltetrazolium bromide] assay. The effect of the treatments on the cell cycle phases was determined by flow cytometry using a Beckman-Coulter FACScan. Apoptosis was measured as the proportion of cells in the sub G₀/G₁ phase of the cell cycle. An assessment of the generation of reactive oxygen species (ROS) after treatments was also carried out. Treatment of cells with indomethacin decreased cell viability and was accompanied by apoptotic DNA fragmentation. Pretreatment of cells with KV significantly suppressed cell death caused by long-term indomethacin treatment. In addition, indomethacin-induced RGM-1 cell apoptosis was evidenced by an increase in the proportion of cells in the sub-G₀/G₁ phase from 6.93 ± 2.56% in the unexposed cells to 30.44 ± 2.56% in cells exposed to 500uM indomethacin (P < 0.001). Treatments with 0.5ug/ml and 5.0ug/ml KV reversed the apoptotic effect of indomethacin. In vivo, pre-treatment of rat with KV significantly reduced indomethacin-induced ulcer formation and total apoptotic score. These results imply that the inhibition of apoptosis may be an important mechanism via which KV protects the stomach from ulcerogenesis.

Keywords: *Indomethacin-induced-apoptosis, ulcer, gastric, RGM-1 cell line*

INTRODUCTION

Gastric mucosal damage occurs as a result of imbalance between aggressive factors such as hydrochloric acid, helicobacter pylori, pepsin and gastrin as well as the

defensive factors such as barrier mucus and bicarbonate. Antiulcer agents therefore act by either increasing the activity of the mucosal defensive or decreasing those of the aggressive factors, or both. Gastric mucosal injury associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most frequent types of gastric mucosal injury (Hawkey 2000; Rainsford, 1993; Schoen and Vender, 1989).

Garcinia kola Heckel (family *Guttiferae*) is a herb grown in Nigeria. It has a characteristic astringent, bitter and resinous taste like raw coffee beans. The seed of this plant called "bitter kola" is highly valued in West Africa for its edibility and use in traditional hospitality. Extracts of various parts of the plant are used extensively in traditional African medicine

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(Ayensu 1978), especially for preparation of remedies for treatment of laryngitis, cough and liver diseases (Iwu 1985). Chemical investigations of seeds of *Garcinia kola* have shown that they contain a complex mixture of phenolic compounds including GB-type biflavonoids, xanthenes, triterpenes and benzophenones, as well as cycloartenol and its 24-methylene derivative (Cotterhill *et al.* 1978). The biflavanones are the most dominant in the *Garcinia* species (Waterman & Hussain 1983).

The crude extracts of the seed of *Garcinia kola* (GK) and its constituent biflavonoid, Kolaviron (KV), have been shown to effectively protect the stomach of rats from ulceration induced by HCl/Ethanol mixture as well as indomethacin (Ibironke *et al.*, 1997; Olaleye, 2005; Olaleye and Farombi, 2006). In these studies, G.K and KV were shown to inhibit *in-situ* gastric acid secretion. However, the mechanism of this inhibition is not known.

Apoptosis has been identified as a potent factor in gastrointestinal epithelial cell turnover as well as ulcerogenesis (Nagano *et al.*, 2005). Furthermore, NSAIDs such as indomethacin have been shown to induce apoptosis in the whole stomach (Imamine *et al.*, 2001) as well as in isolated gastric cells in culture. For instance, Tomisato *et al.* (2004) reported that guinea pig gastric mucosal cells in primary culture treated with low concentrations of indomethacin decreased cell viability accompanied by apoptotic DNA fragmentation, chromatin condensation, and caspase activation.

Another factor that plays a critical role in the generation of gastric and duodenal ulcers is production of reactive oxygen species (ROS). This is evidenced by the increased oxidative stress by pro-ulcerative factors in the gut such as *Helicobacter pylori* (Uemura *et al.*, 2001), use of non-steroidal anti-inflammatory drugs (Soll *et al.*, 1991), Smoking, psychological stress, corticosteroid use (Levenstein, 1996) and loss of sleep. Lipid peroxidation (LPO), a result of the reaction of oxyradicals and polyunsaturated acids, has been suggested as an attack factor in the gastric mucosa. Specifically, ROS generation has been associated with apoptosis induced by indomethacin and other non-steroidal anti-inflammatory drugs in cultured gastric cells (Imamine, 2001). In previous studies, KV has been shown to prevent lipid peroxidation induced by various agents in the liver (Farombi, 2000) and stomach (Olaleye and Farombi, 2006). It is therefore reasonable to suggest that the ability of KV to inhibit ROS production may contribute to its gastroprotective action.

In the present study the effects of KV on indomethacin-induced ulceration and apoptosis was

studied in intact rat stomach and the rat gastric mucosal cells (RGM-1)

MATERIALS AND METHODS

Plant material

Seeds of *Garcinia kola* Heckel (Family Guttiferae) were collected from cultivated plants in a homestead in Ibadan, Nigeria. The identity of the plants was confirmed by Professor A. Egunyomi of the Department of Botany, University of Ibadan. A voucher specimen is available in the herbarium of the same institution. Peeled seeds (5 kg) were sliced, pulverized with an electric blender and dried at 40 °C in a Gallenkamp drying oven.

Preparation of kolaviron

Kolaviron was extracted from the powdered seeds of *Garcinia kola* according to the previously described method (Farombi *et al.*, 2000). Briefly, the powdered form of *Garcinia kola* seeds (4 kg) was extracted with light petroleum ether (b. pt 40–60 °C) in a soxhlet for 24 h. The defatted, dried marc was extracted with acetone. The extract was concentrated and diluted with twice its volume of water and extracted with ethyl acetate (6 × 300 mL).

The concentrated ethyl acetate fraction gave a yellow solid known as kolaviron (165 g), which has been shown to consist of *Garcinia biflavanones* GB-1, GB-2 and kolaflavanone (Iwu, 1985).

In-Vitro Studies

Cell culture/treatments

Rat gastric mucosal epithelial cell line RGM-1 (RCB-0876, Riken Cell Bank, Tsukuba, Japan), a diploid, non-transformed epithelial line isolated from normal Wistar rat's gastric mucosa (Kobayashi *et al.*, 1996) was used for this study. The cells were grown in DMEM/F-12 medium containing 20% heat-inactivated, fetal bovine serum and supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin, and 20% FBS in an incubator at 37°C, 95% humidity, and 5% CO₂. Cells were routinely subcultured in 15ml medium in Iwaki flasks using trypsin/EDTA. Cultures were examined regularly under an inverted light microscope to monitor growth and rule out contamination. For this study, data for passages 9 – 22 were found to be consistent and were thus used. For treatment of the RGM-1 cell, the KV was first diluted in DMSO. Further dilution was made with tissue culture media

and then added to cultured cells to give the final indicated doses. In control experiments, DMSO alone was diluted with tissue culture media. At the specified times, control and treated cells were harvested.

Cell viability:

Cell viability was determined by the MTT [3-(4, 5 – dimethylthiazol-2-yl)-2,5-deiphenyltetrazolium bromide] assay, by measuring the conversion of MTT tetrazolium salt to MTT formazan. Yellowish MTT stock solution was prepared using phosphate-buffered saline (PBS). Further dilution was made with the medium to give concentrations of 5 mg/ml DMEM/F12. This was added to each dish (in triplicate for each assay condition) at a volume of 10% of the original culture volume and incubated for 3 h at 37 °C in humidified CO₂. At the end of the incubation period the medium was removed, and the blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol). MTT conversion to formazan by metabolically. The absorbance was measured at 570 nm in an enzyme-linked immunosorbent assay microplate reader (iQuant, Bio-Tek Instruments, Inc., Vt., USA). The OD value was positively related to the cell survival. A decrease in optical density compared with the control cells provided a quantitative assessment of cell death (Centeno *et al*, 1998).

Apoptosis study:

The Apoptotic rate was studied in the rat gastric mucosal (RGM1) cell line using flow cytometry. Briefly, 2 X 10⁴ cells were seeded in a 6-well Iwaki culture plate and allowed to reach 60% confluency. After washing the cells with PBS, The cells were then starved for 48hours to synchronize the cells at G₀/G₁ phase. Thereafter, the cells were challenged with medium containing 500uM indomethacin in the presence or absence of 0.5ug/ml and 5.0ug/ml KV.

After 24h of incubation, cells were harvested and washed with PBS, resuspended in 500 µl of PBS, and fixed in 500 µl of ice-cold absolute ethanol at –20 °C. After incubation for 30 min, cell pellets were collected by centrifugation, re-suspended in 0.5 ml of PBS containing 100 µg/ml RNase, and incubated at 37 °C for 30 min. Then 0.5 ml of propidium iodide (PI) solution (50 µg/ml in PBS) was added, and the mixture was allowed to stain on ice for 60 min. The effect of the treatments on the cell cycle phases was determined by flow cytometry using a Beckman-Coulter FACScan. For each experiment, a total of 10⁴ cells were counted.

Analysis of the flow cytometry result was done using Cychred and WinMDI (Purdue University) software. Apoptosis was measured as the proportion of cells in the sub G₀/G₁ phase of the cell cycle.

In-Vivo Studies

Animals:

Male albino rats of the Wistar strain weighing 150 - 200g were obtained from the central animal house, College of medicine, University of Ibadan. The rats were acclimatized to the animal house conditions (light and dark cycle 12:12 and temperature 26 ± 1⁰C) and had free access to food (Ladokun feeds Ltd, Ibadan, Nigeria) and water *ad libitum*.

Indomethacin-induced Gastric Ulceration:

After a 24-h fast (but with water ad-libitum), a group of rats were assigned to receive indomethacin (40mg/kg, dissolved in 5% w/v Sodium bicarbonate solution, pH 8.0) Two groups received 20mg/kg and 100mg/kg of Kolaviron respectively for 3 days prior to indomethacin administration. A control group received an equivalent volume of 5% w/v Sodium bicarbonate solution.

4-h after indomethacin administration, the animals were sacrificed by ether overdose. The stomachs were opened along the greater curvature, washed in normal saline and pinned on a cork for ulcer scoring. Macroscopically visible ulcers were independently scored by two observers unaware of the treatments. The total ulcer score was calculated as the sum of the lengths of all gastric mucosal injuries (in mm)

Staining for Apoptosis:

Samples of the gastric mucosa were removed and immersed in 10% formalin. After embedding in paraffin, 4um sections were mounted on glass slides and stained using haematoxylin eosin. Counting of apoptotic bodies was done using a computer-aided digital camera fixed to a microscope. Apoptotic score was defined as the number of apoptotic bodies/2,000 cells

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Student's *t*-test (unpaired) was used to compare data between two groups using the Microsoft Excel[®] software. The level of significance was set at *P* = 0.05

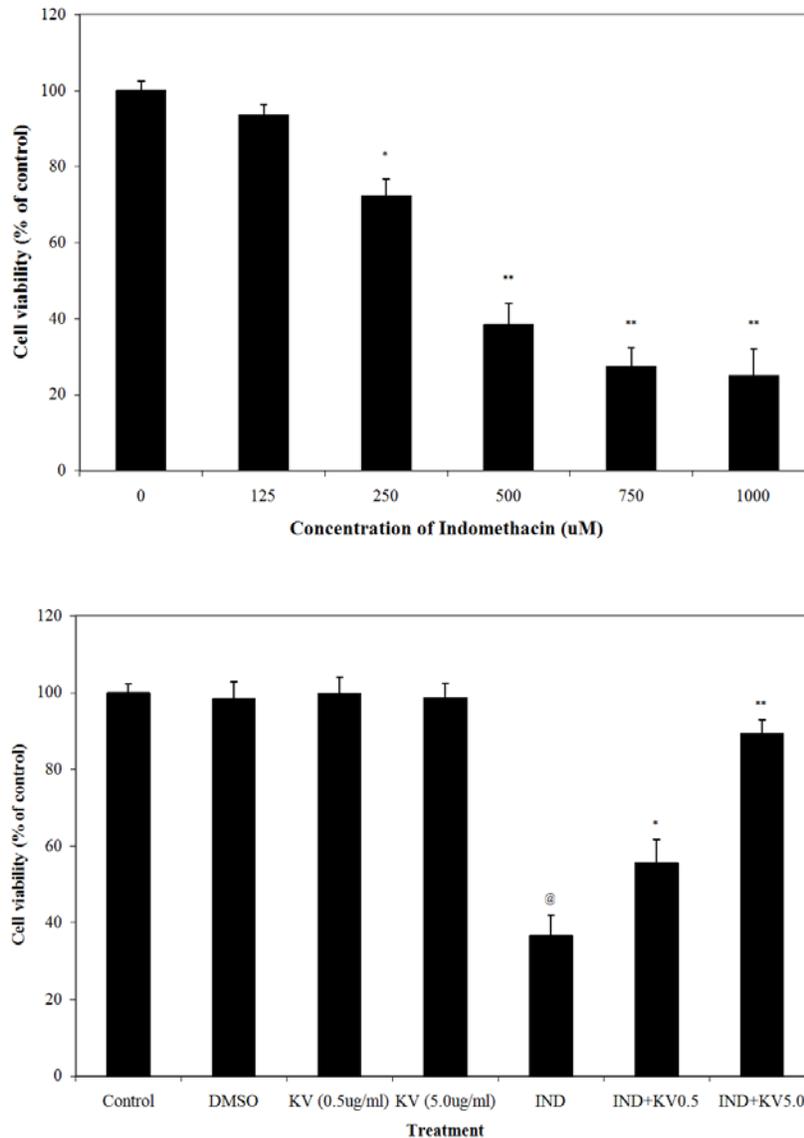


Fig. 1

(A) Effect of graded concentrations of indomethacin on the viability of RGM-1 cells. (B) Effect of pre-treatment of RGM-1 cells with kolaviron (0.5ug/ml and 5.0ug/ml) on viability. Each vertical bar represents mean \pm SEM of three experiments in triplicate. @p < 0.01 (c.f. DMSO treated group), *p < 0.05 and **p < 0.01 (c.f. indomethacin treated group)

RESULTS

Cell viability studies

Fig. 1a shows the effect of graded concentrations of indomethacin on the viability of gastric mucosal cells. There was a graded increase in cell death as the concentration of indomethacin increases, the viability becoming significant from 250uM. For the subsequent experiments an indomethacin concentration of 500uM was used.

The results of treatments with kolaviron are presented in Fig. 1b. 500uM of indomethacin significantly decreased cell viability to $36.74 \pm 5.22\%$ (c.f. $100 \pm 2.25\%$ in the control cells. Pre-treatment with KV significantly reversed the decrease in cell viability induced by indomethacin, the mean cell viabilities being $55.62 \pm 5.11\%$ and $89.27 \pm 3.73\%$ for the groups pre-treated with 0.5ug/ml and 5.0ug/ml KV respectively.

Table 1

Apoptosis in control RGM-1 cell line and those treated with indomethacin in the presence or absence of 0.5ug/ml and 5.0ug/ml of Kolaviron.

Treatment	Dose	Apoptotic Score*	Percentage of Cells in Apoptosis phase**
Control (0.1% DMEM)	0	6.93 ± 2.56	2.59 ± 0.30
DMSO	<0.01%	6.55 ± 4.22	2.49 ± 0.98
Kolaviron	0.5ug/ml	6.88 ± 2.11	2.55 ± 0.75
	5.0ug/ml	7.06 ± 1.59	2.92 ± 0.61
Indomethacin	500uM	30.44 ± 2.56***	19.65 ± 2.83***
Kolaviron + Indomethacin	KV (0.5ug/ml) + IND (500uM)	20.64 ± 4.63	10.96 ± 0.18**
	KV (5.0ug/ml) + IND (500uM)	13.88 ± 2.51	5.34 ± 1.33*

Values are expressed as Mean ± SEM of 6 experiments. *Apoptotic score (obtained from Cychred Cell cycle analysis software (Purdue University) was calculated as the total number of cells in the sub G0/G1 Phase divided by 10,000. ** Obtained from the dotplot via the winMDI software (Purdue University)

Table 2

Indomethacin-induced apoptosis in Control and Kolaviron pre-treated animals. Values are mean ± SEM of n.

Group	Treatment	Total Ulcer Score	Apoptotic score
1	5% NaHCO ₃	-	1.60 ± 0.20
2	40mg/kg indomethacin in 5% w/v NaHCO ₃	23.3 ± 4.20	18.20 ± 2.10
3	KV (20mg/kg) + 40mg/kg indomethacin in 5% w/v NaHCO ₃	16.20 ± 3.30	11.50 ± 3.20
4	KV (100mg/kg) + 40mg/kg indomethacin in 5% w/v NaHCO ₃	8.70 ± 2.10	4.80 ± 0.80

Effect of KV on indomethacin-induced apoptosis in RGM-1 cells

Indomethacin has been previously shown to be capable of inducing both apoptosis and necrosis in RGM-1 cells. The flow cytometry study was therefore carried out to ascertain the type of cell death.

The results of the flow cytometry is shown in Table 1 while the dotplot obtained from a well each for the treatments are shown in Fig. 2. Indomethacin at a dose of 500uM significantly induced apoptosis in the gastric epithelial cells. This is consistent with previous findings and is illustrated by the high density of the Propidium iodide staining for the apoptotic cells. Pre-treatment of the cells with 0.5ug/ml and 5ug/ml of kolaviron significantly reduced the apoptosis induced by indomethacin.

Effect of KV on indomethacin-induced ulceration and apoptosis in rats

Table 2 shows the ulcer and apoptotic scores in the gastric epithelial cells in control and indomethacin-treated rats with or without KV pre-treatment. Indomethacin (40mg/kg) induced ulcers in all the rats (ulcer score = 23.3 ± 4.2). No visible ulceration was observed in the control rats. Pre-treatment with KV (20mg/Kg and 100mg/kg) significantly reduced ulcer induced by indomethacin (ulcer scores were 18.20 ± 3.7 and 8.70 ± 1.60 respectively). The number of apoptotic bodies counted per 2000 cells in indomethacin-treated rats (18.20 ± 2.10) significantly higher than the number observed in the animals with KV pre-treatment. Mean apoptotic bodies in controls were 11.50 ± 3.20 and 4.80 ± 0.90 respectively (p < 0.05, c.f. indomethacin-treated group)

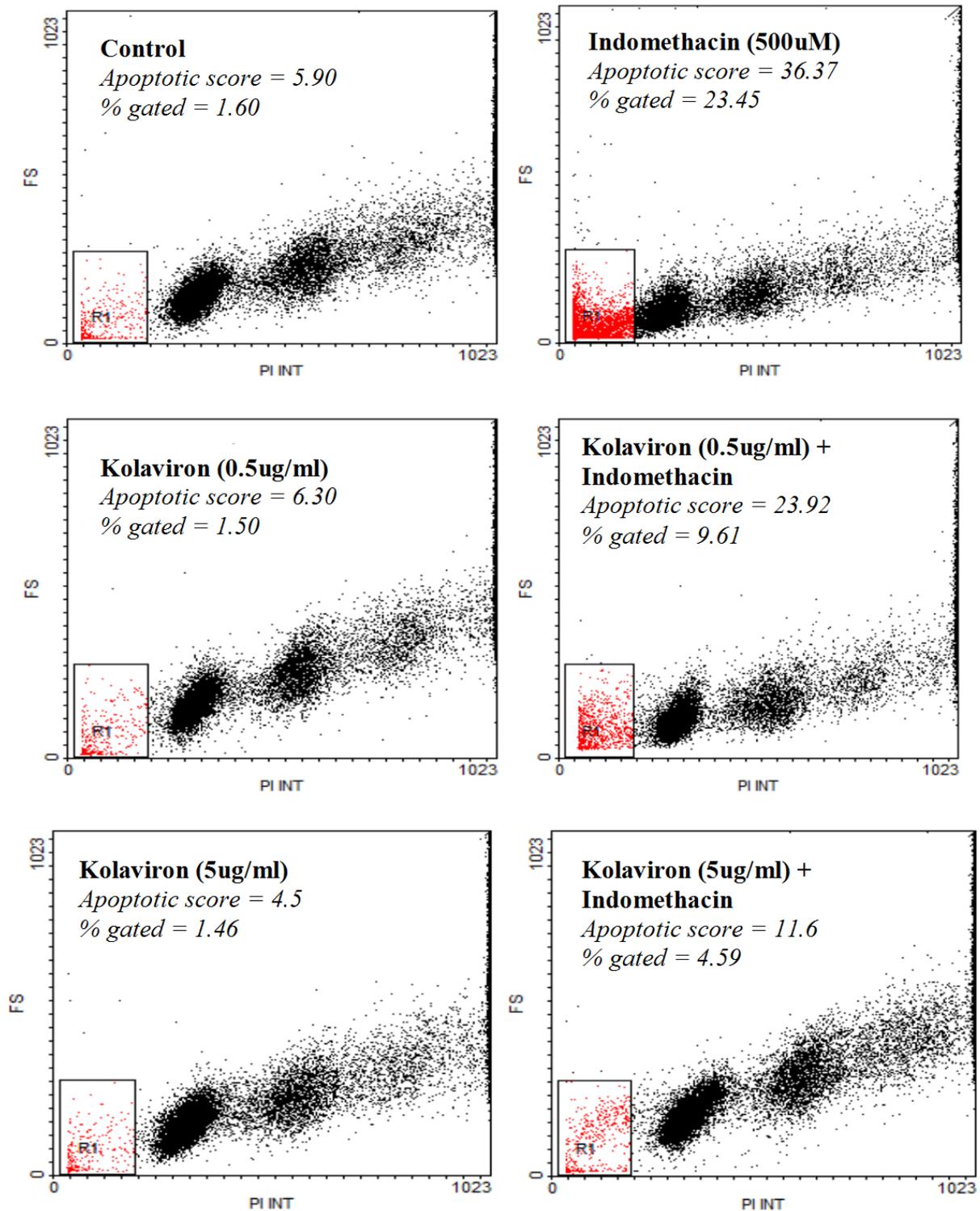


Fig. 2

Representative flow cytometry dotplots for control, indomethacin and Kolaviron-treated Rat Gastric Mucosal (RGM-1) cells. The percentage of cells in the gated areas are as indicated.

DISCUSSION

In this study, the ability of Kolaviron, a biflavaroid fraction from the seed of *Garcinia kola* to inhibit apoptosis induced by a non-steroidal anti-inflammatory drug (NSAID) indomethacin was evaluated using whole animal and cultured gastric mucosal cells. The results show that treatment of intact rats with indomethacin significantly produced visible gastroduodenal damage 4 hours after treatment. This confirms earlier observation on the effects of indomethacin on gastric mucosal cell in rats (Yoshikawa *et al.*, 1994) and humans Maits *et al.*, 1998).

Several studies agree that NSAIDs are very important in the etiology of gastrointestinal lesions (Allison *et al.*, 1992; Rainsford, 1993) despite their being effective in the management of chronic conditions such as rheumatoid arthritis. Many theories have also been used to explain the gastric injury caused by indomethacin and other NSAIDs, including inhibition of prostaglandin synthesis (Skarstein, 1979; Tarnawski, 1988; Vane, 1996), impairment of gastric mucosal blood flow (Wang *et al.*, 1989; Hirose *et al.*, 1991), increased gastric acid secretion (Okcu *et al.*, 1992; Schmann and Switzerland, 1998), direct action on gastric epithelial cells (Tarnawski, 1988; Tomisato *et al.*, 2004), reduction in mucus and bicarbonate secretion (Baumgartner *et al.*, 2004; Jarwosky *et al.*, 2005).

Lately, it has been shown that increased apoptotic cell death and simultaneous block of mucosal cell renewal play major roles in the development of mucosal lesion by indomethacin. Healthy gastric mucosa is always under equilibrium between cell death and cell renewal (Jones *et al.*, 1999; Konturek *et al.*, 1999; Imamine *et al.*, 2001). Thus ulcer develops when there is preponderance of cell death over cell proliferation or inhibition of cell proliferation. The induction of apoptosis in the stomach by NSAIDs, including indomethacin, occurs via reactive oxygen species generation (Wallace *et al.*, 1993; Takeuchi *et al.*, 1996), cytochrome *c* release, activation of caspase-3, inhibition of survivin expression, and induction of calcium signaling (Asako *et al.*, 1992; Appleyard *et al.*, 1996).

The result also show that pre-treatment of intact rats with Kolaviron significantly inhibited the increased ulcer score by indomethacin. Kolaviron in its pure form or as crude extract (*Garcinia kola*) has been shown in previous studies to attenuate gastrointestinal injuries induced by several ulcerogens such as indomethacin (Ibironke *et al.*, 1997), ethanol (Olaleye, 2005) and HCl-Ethanol mixture (Olaleye and Farombi, 2006). Attempts have been made to postulate a mechanism for the gastroprotective effects of Kolaviron. Rats fed diets containing seeds of *Garcinia kola* were reported to have

reduced gastric acid secretion when compared to the untreated controls (Ibironke *et al.*, 1997), thus underscoring the role of gastric acid. The finding that kolaviron significantly reversed the increase in reactive oxygen species (Olaleye and Farombi, 2006) led to the idea of the present work.

In summary, the results of this study corroborated previous reports that administration of indomethacin resulted induction of gastric mucosal injury and apoptosis in intact rats and primary cultures of gastric epithelial cells. Pre-treatment with kolaviron attenuated the pro-apoptotic effects of indomethacin. This suggests that inhibition of apoptosis may be an important mechanism via which KV protects the stomach from ulcerogenesis..

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