Effect of Reserpine on the Expression of Tumour Promoter, Tumour Suppressor and Ulcer Healing Genes in the Stomach of Female Sprague-Dawley Rats

Isyraqiah Faizatul\(^1\), Methil Kannan Kutty\(^1\), Damayanthi Durairajanayagam\(^1\), Norita Salim\(^1\), Sergey Gupalo\(^1\), Norizan Kamal Basah\(^1\), Harbindar Jeet Singh\(^{1,2,3}\)

ABSTRACT

Introduction: Reserpine is often used to induce gastric ulcers in animal models but has also been shown to induce tumours of adrenal and mammary glands. However, its effect on the expression of tumour promoter, suppressor, and ulcer healing genes in the stomach is unknown.

Aims: This study examined the expression of these genes in the stomach of rats 24 hours after reserpine treatment.

Materials and Methods: After an overnight fast, sixteen, 6-week old female rats were divided equally into control and experimental groups (20 mg/kg of reserpine intraperitoneally). All rats were euthanized after 24 hours. Body weights were measured before treatment and 24 hours after treatment. Following euthanization, their stomachs were collected for histopathological examination, and expressions of tumour promoter (L-myc, PGC), tumour suppressor (ARID1A, E-Cadherin, FAT4, APC), and ulcer healing genes (PPARG, TFF1, TFF2) using RT-qPCR. Data were analysed using one-way ANOVA.

Results: Haemorrhagic gastric ulcer developed after 24 hours of reserpine treatment. Microscopy showed superficial ulceration with degenerated necrotic mucosa and inflammatory cells. Expression of E-cadherin, APC, FAT4, PGC, Lmyc, and TFF1 genes was significantly lower, whereas ARID1A and TFF2 expressions were significantly higher in reserpine-treated rats. No changes were observed in the expression of PPARG. Body weight of reserpine-treated rats was significantly lower compared to that in controls.

Conclusions: The decreased expression of E-cadherin, APC, FAT4, and TFF1 suggests that reserpine might promote gastric carcinogenesis. However, other tumour promoter genes need to be examined to ascertain the effect of reserpine on gastric carcinogenesis.

KEY WORDS

reserpine, gastric ulcer, tumour promoter, tumour suppressor, ulcer healing

INTRODUCTION AND LITERATURE REVIEW

Reserpine is well-known for its role as an anti-psychotic\(^1\) and anti-hypertensive drug\(^2\), although it is rarely used today to treat hypertension due to its many side effects\(^3\). Reserpine has however been used successfully to treat patients with psychosis and mania\(^4\). As a monoamine depletor, reserpine has been used as a model to induce depression in studies investigating anti-depressant drugs\(^5\). Repeated low dose of reserpine administration leads to changes in motor functions accompanied by higher striatal oxidative stress, and has therefore been suggested as a viable rat model for the neuroprogression of Parkinson’s disease\(^6,7\).

However, administration of high doses of reserpine has also been shown to cause adverse reactions, particularly in inducing gastric ulcers\(^8\). Studies on reserpine-induced gastric ulcers have been well documented, and it has been proven to be a good toxic chemical for studies related to gastric ulcer\(^9,10\).

Long-term exposure to low doses of reserpine was found to increase the incidence of adrenal medullary pheochromocytomas, undifferentiated carcinomas of seminal vesicles, and mammary cancers in rats and mice\(^11\). Although reserpine is often used as an ulcer causing agent, no reports exist on its effects on tumour related genes. It is generally known that tumorigenesis involves the down-regulation of tumour suppressor genes and up-regulation of tumour promoter genes. Many stud-
ies have depicted the role of E-cadherin (Epithelial Cadherin), APC (Adenomatous Polyposis Coli), ARID1A (AT Rich Interactive Domain 1A), and FAT4 (FAT Atypical Cadherin 4) genes as tumour suppressors in gastric cancers\(^{15-18}\). PGC (pepsinogen C) and L-myc (Myelocytomatisis) genes that function as tumour promoters are also described in gastric cancer studies\(^{19,20}\). In addition, TFF1 (Trefoil factor 1), TFF2 (Trefoil factor 2), and PPARG (Peroxisome Proliferator-activated receptor gamma) have been reported to play an important role in the healing of gastric ulcers\(^{21-23}\). Hence, the aim of this study was to evaluate the effect of reserpine on the expressions of tumour promoter, tumour suppressor, and ulcer healing genes in normal rats.

**MATERIALS AND METHODS**

**Animals and diets**

Sixteen, six-week old female Sprague-Dawley rats (135 - 145 g) were obtained from the Laboratory Animal Care Unit (LACU), Faculty of Medicine, Universiti Teknologi MARA (UiTM) Sungai Buloh, (Selangor, Malaysia). The rats were housed in polypropylene cages, with a stainless steel wire grid as cover, in the animal room of LACU. Ambient temperature and relative humidity were maintained at 23 - 25\(^\circ\)C and 50 - 55 % respectively, with a 12-hour light/dark cycle. Their beddings were changed twice a week. The rats had access ad libitum to vegetarian diet pellet (Special Feeds, Australia) and reverse-osmosis (RO) drinking water. Animal handling and experimental treatment procedures were done in accordance with the Guidelines for Care and Use of Laboratory Animals prepared by the Animal Care and Users Committee (ACUC, UiTM Shah Alam, Selangor, Malaysia). This study was approved by the Animal care and users Committee of the Faculty of Medicine, UiTM.

**Experimental animals and grouping**

Briefly, sixteen female rats were divided into two groups (Group 1 and 2), consisting of 8 rats per group. All rats were fasted 24 hours before the start of the experiment. This was labelled as day 0. Group 1 was kept as controls, while Group 2 was administered with a stat dose of 0.1 mL of 20 mg/kg reserpine suspended in 0.5% acetic acid solution via intraperitoneal route\(^{24}\). This was labelled as day 1. All rats were again fasted for another 24 hours (day 2). Then, they were lightly anesthetized with diethyl ether and euthanized using a small guillotine. The rats were then viewed under a light microscope at 200x and 400x magnifications.

**Histological Study**

Stomachs of rats were collected and sectioned longitudinally, dividing the lesser and greater curvatures. One part of the stomach was fixed in 10% neutral buffered formalin and processed before being embedded in paraffin. Thin sections were made and stained with haematoxylin and eosin (HE). The slides were then viewed under a light microscope at 200x and 400x magnifications.

**RT-qPCR Analysis**

The other part of the stomach was stored at -80\(^\circ\)C immediately after dissection. InnuPREP RNA Mini Kit (Analytik Jena, Germany) was used to extract RNA from the stomach tissues and were stored immediately at -20\(^\circ\)C. The RNA extract was then converted to cDNA using Maxima First Strand cDNA synthesis kit (Thermo Scientific, USA) and incubation was performed using Mastercycler pro thermal-cycler (Eppendorf, Germany). The cDNA was then stored at -20\(^\circ\)C. Reverse-Transcriptase quantitative PCR (RT-qPCR) was performed to determine the expressions of tumour suppressor genes (FAT4, ARID1A, APC, E-Cadherin), tumour promoter genes (PGC and L-myc), and ulcer healing genes (PPARG, TFF1, and TFF2) using Luminaris Colour HiGreen qPCR Master Mix (2X) (Thermo Scientific, USA). RT-qPCR was run using PCR thermal cycler CFX 96 (Bio-Rad, USA). GAPDH and RPL29 was used as reference genes to normalize the relative gene expressions.
Statistical analysis

Body weight was analysed using two-way ANOVA, while gene expression was analysed using one-way ANOVA. A 'p' value of less than 0.05 was considered statistically significant.

RESULTS

No significant differences were evident in body weight between the two groups at day 1. Body weight of reserpine-treated rats was significantly lower compared to that of the control group at day 2 (p < 0.001) (Figure 2). Twenty-four hours after reserpine administration rats were found to bleed from the nostrils and eyes (Figure 3). Reserpine-treated rats developed haemorrhagic ulcers in the stomachs (Figure 4). Microscopic examination of the ulcerated stomach showed degenerated necrotic mucosa and presence of inflammatory cells (Figure 5). In the reserpine-treated group, the expressions of E-Cadherin (p < 0.05), FAT4 (p < 0.001), and APC (p < 0.001) were significantly lower, while expression of ARID1A was significantly higher (p < 0.001) when compared to those in the controls (Figure 6.1). Reserpine-treated rats had significantly lower expressions of L-myc and PGC in the stomach when compared to that in the controls (Figure 6.2). Reserpine-treated rats had significantly lower expression of TFF1 (p < 0.001) and significantly higher expression of TFF2 (p < 0.001) when compared to that in the controls (Figure 6.3).

DISCUSSION

Reserpine induced acute haemorrhagic ulceration of the gastric mucosa at the greater curvature near the antral region (Figure 4). Histopathological examination of the ulcer revealed an area of degenerated necrotic mucosa (Figure 5). Inflammatory cells were also observed near the ulcerated area (Figure 5). Induction of gastric ulcer by reserpine has been reported before, where it becomes evident from as early as 18 hours after its administration, provided the animals were fasted for 24 or 36 hours prior its administration25). Reserpine most probably triggers gastric ulceration through indirect over-stimulation of the vagus nerve. The direct effect of reserpine as a monoamine depletor might be at the sympathetic neurons first, which then causes an increase in the activity of the parasympathetic neurons, including the vagus nerve. Reserpine is reported to prevent the accumulation of catecholamines in the synaptic vesicles of sympathetic neurons by competing with catecholamines for the binding site on the vesicular membrane transporter 2 (VMAT2) receptor, binding irreversibly to the receptor26). The inhibitory effect of reserpine in the sympathetic neurons results in an over-stimulation of the vagus nerve in the stomach, which then releases acetylcholine that binds to its receptors at the parietal cells. Diffusion of calcium ions increases, which then activates phosphorylation of kinases. The proton pump is then activated, secreting hydrogen ions into the stomach. Excessive gastric acid secretion then induces the development of gastric mucosal lesions and ulcers27,28).

Reserpine-treated rats had significantly lower expression of tumour suppressor E-cadherin, FAT4, and APC compared to control (Figure 6.1). To our knowledge, this is the first study reporting the effect of reserpine on the expressions of tumour promoter, tumour suppressor, and ulcer healing genes. E-cadherin plays a role in cell-to-cell adhesion between epithelial cells. Each cadherin forms an intersection with a cadherin on an adjacent cell, which forms an adheren junction29). Inactivation of E-cadherin reduces cellular adhesion, promoting metastasis of malignant tumour cells30). E-cadherin expression has been reported to be significantly lower in patients with gastric adenocarcinoma15). Low expression of E-cadherin might promote tumorigenesis in reserpine-treated rats. FAT4 gene plays a role in regulating planar cell polarity of epithelial cells31,32). It synthesizes transmembrane proteins that arrange and align specialized cells; like body hair in one specific direction through asymmetric localization32,33). Loss of FAT4 protein function promotes the development of hyperplastic epithelial cancer cells34-36). Patients with gastric cancer with poor 5-year disease survival rate have been shown to have significantly lower FAT4 expression37). Low expression of E-cadherin might promote tumorigenesis in reserpine-treated rats. APC gene is an important tumour suppressor, and about 80% of colon cancers have APC mutations. It regulates the ubiquitination and disintegration of oncogene B-catenin. APC inactivation leads to accu-
mulation of B-catenin, which subsequently triggers transcription of cyclin D1 and C-myc\textsuperscript{40,41}. Patients with intestinal type gastric cancer exhibited mutations in the APC gene\textsuperscript{37,38}. Low expression of APC in reserpine-treated rats might promote gastric cancer. However, reserpine-treated rats had significantly higher expression of ARID1A compared to controls, indicating that reserpine plays a role in regulating expressions of genes through remodelling of chromatin structure. High expression of ARID1A causes chromatin to bind tightly, hence lowering the activities of gene transcriptions, replications, methylations, and repairs. Silencing of ARID1A gene causes unnecessary transcription of genes that are involved in stimulating proliferation of cells\textsuperscript{42}. Patients with gastric cancer show higher expression of ARID1A compared to their matched non-tumour tissues\textsuperscript{39}. Reserpine might increase ARID1A expression to suppress the development of gastric cancer. Reserpine-induced ulcer might promote gastric carcinogenesis by lowering the expression of tumour suppressors, which might explain the increase risk of developing gastric cancer among patients with gastric ulcer\textsuperscript{43}. This is somewhat similar to that in this study, where the expression of PGC was significantly lower in reserpine-treated rats. It is possible that PGC might be involved in the development of cancer types because PGC might alternatively be used as a biomarker for gastric cancer\textsuperscript{44}. Myc gene is an oncogene that encodes a transcription factor protein\textsuperscript{45}. It binds to specific genes and activates its transcription, which may be involved in cell cycle, apoptosis and cell differentiation\textsuperscript{46}. Mutation of Myc gene is found in about 50% of Burkitt's lymphoma patients\textsuperscript{47}. It regulates Bcl-2, which inhibits apoptosis of B-cell lymphomas\textsuperscript{48}. Myc protein was found over-expressed in gastric cancer patients, and the promoter Myc gene was hypo-methylated\textsuperscript{49}. It is possible that L-myc, a variant of Myc, might not be involved in gastric cancer development. L-myc is highly expressed in small cell lung carcinomas, but C-myc; another variant of Myc is highly expressed in solid tumours. C-myc was found highly expressed in gastric cancer cells, and may involve in promoting its growth\textsuperscript{50}. Reserpine might lower the expression of L-myc to inhibit lung carcinoma development, but the role of C-myc in promoting gastric carcinogenesis in reserpine-treated animals needs to be investigated. Gastric carcinogenesis may not involve tumour promoter L-myc and PGC in reserpine-treated rats. No significant difference was evident in the expression of PPARG in reserpine-treated rats compared to that in the control (Figure 6.3). PPARG is a ligand-activated transcription factor found in genes involved in energy homeostasis and lipid metabolism\textsuperscript{51,52}. If PPARG by its agonist troglitazone inhibits the proliferation of gastric cancer cells, possibly by favouring apoptosis and causing the arrest of G1 cell cycle\textsuperscript{53}. However, in reserpine-treated rats, no significant changes were evident in PPARG expression, which probably indicates that PPARG does not play a role in gastric carcinogenesis in reserpine-treated rats. Perhaps if the treatment duration were longer, the expression of PPARG would be significantly lower, hence indicating that reserpine might lower the expression of PPARG to promote gastric carcinogenesis. In addition, the expression of TFF1 was significantly lower in reserpine-treated rats (Figure 6.3). TFF1 proteins are secreted by the pit cells of stomach, and plays a role with mucin in maintaining the stomach’s integrity by forming the mucous barrier\textsuperscript{54}. Expression of TFF1 was significantly higher in patients with gastric ulcers, and was related to the gastric ulcer healing process\textsuperscript{55}. However, TFF1 expression was down-regulated in gastric cancer cell lines due to hyper-methylation of its promoter gene\textsuperscript{56}. Reserpine may promote gastric carcinogenesis by lowering the expression of TFF1 in the stomach. The expression of TFF2 was significantly higher in the reserpine-treated group compared to that in the control group (Figure 6.3). TFF2 is secreted by mucus neck cells of gastric glands. TFF2 and is important in the recovery of damaged gastric mucosa\textsuperscript{57,58}. TFF2 gene was hypo-methylated in patients with gastric ulcer\textsuperscript{59}. TFF2-deficient mice secreted higher levels of gastric acid, low number of mucus cells, and had thin mucosal layer, which might be due to lower proliferative rate of the mucus cells\textsuperscript{60}. However, hyper-methylation of TFF2 promoter gene was found to significantly lower the expression of TFF2 in gastric cancer cell lines\textsuperscript{61}. Reserpine may increase the expression of TFF2 to promote healing of the ulcerated mucosa based on the presence of inflammatory cells. However, TFF2 may not be involved in gastric carcinogenesis in reserpine-treated rats.
Expression of Genes in the Stomach


