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Research Article

LOW LEVELS OF MICROVESSEL DENSITY AND IMMUNOHISTOCHEMICAL EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN CARCINOGEN-INDUCED DUCTAL MAMMARY GLAND CARCINOMA OF RATS SUPPLEMENTED WITH GARLIC

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ABSTRACT

The present study was conducted to evaluate the anti-angiogenesis activity of diet containing 5% of garlic powder (G) on mammary gland carcinoma induced by *N*-methyl-*N*-nitrosourea (MNU) in female Sprague-Dawley® rats. A total of 24 female Sprague-Dawley® rats were randomly assigned into three groups, namely control, MNU, and MNU+G (n=8). MNU group rats received four consecutive subcutaneous (SU) injections of MNU at a dose of 60 mg/kg per injection and basal diet, MNU+G group rats received four consecutive SU injections of MNU at a dose of 60 mg/kg per injection and diet containing 5% of garlic powder, while control rats were injected with normal saline and fed with normal diet. All rats were euthanized at 24 weeks of experimental period. Microvessel density (MVD) and vascular endothelial growth factor (VEGF) expressions were investigated through histopathology and immunohistochemistry methods. Results showed MNU-induced ductal mammary gland carcinoma in rats of MNU and MNU+G groups. MVD scoring values showed significant ($p<0.05$) higher score of MNU group compared to MNU+G and control groups. Overexpression of VEGF was observed significantly ($p<0.05$) in MNU group compared to MNU+G and control groups. Administration of diet containing 5% of garlic powder to female Sprague-Dawley® rats minimized the development of ductal mammary gland carcinoma by inhibiting the angiogenesis activity.

Keywords: Ductal mammary gland carcinoma, Microvessel density, Vascular endothelial growth factor, Garlic powder, *N*-methyl-*N*-nitrosourea.

INTRODUCTION

Mammary gland carcinoma is among the most frequent malignant diseases in the world and it is the leading cause of premature death among younger females in developed countries. One of every seven women in Europe has this disease in their lifetime [1]. Angiogenesis is a process of new microvasculature formation. Angiogenesis is a very important process in the growth and metastasis of malignant tumors. Tumor microvessel density (MVD) plays a significant role in mammary gland carcinoma development, where advanced stage of mammary gland carcinoma showed high MVD tumor [2]. High MVD is observed with overexpression of vascular endothelial growth factor (VEGF) [3]. VEGF is a protein that plays a main role in tumor angiogenesis [4,5]. VEGF expression increased collaterally with histological progression in invasive mammary gland carcinoma (4). Apart from that, serum VEGF levels were also reported to be elevated in patients with invasive mammary gland carcinoma in comparison with benign mammary gland tumors [4,7].

N-methyl-*N*-nitrosourea (MNU) is one of the carcinogenic agents that were used for the induction of mammary gland cancer in rats [8]. Several studies have indicated that MNU has the ability to induce mammary gland carcinoma in rats by intraperitoneal, subcutaneous (SC), or intravenous routes at different doses and frequencies [9-12].

Garlic is a famous medicinal herb, which exhibits many medicinal properties such as anticancer, antibacterial, antifungal, antivirus, immunostimulating, and antioxidant [13-18]. Garlic reduces the development of mammary gland cancer in animals [19]. Liu *et al*. demonstrated that garlic powder at a dose of 2% of feed intake reduced mammary gland carcinoma induced by 7,12-Dimethylbenz(a)anthracene in rats [20]. The mechanism of garlic anticancer activity was by certain paths including inducing programed cell death, cell cycle arrest, or inhibition angiogenesis and metastasis [21]. Garlic and its compounds showed the ability to inhibit angiogenesis through inhibiting VEFG expression. *In vitro*, aged garlic extract suppressed the proliferation of transformed rat endothelial cell line, reduced the invasiveness of the endothelial cells by about 20-30%, and reduced capillary-like tube formation by the endothelial cells [22]. The diallyl sulphide, diallyl disulfide, and diallyl trisulfide treatments were able to significantly disrupt the capillary-like tube formation and migration by human umbilical vein endothelial cell (HUVEC) that was accompanied by the suppression of VEGF secretion and down regulation of VEGF-receptor 2 expression [23]. Besides, Alliin was also shown to significantly reduce VEGF and fibroblast growth factor-2-induced tube formation and angiogenesis in HUVEC and *ex vivo* [24]. The present study was conducted to investigate the anti-angiogenesis activity of diet containing 5% of garlic powder on mammary gland carcinoma induced using MNU in female Sprague-Dawley® rats.

METHODS

Animals

The use of animals in this study was approved by the Animal Ethics Committee, Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. A total of 24 8-week-old female Sprague-Dawley® rats with body weights of 170-200 g were obtained from local supplier (Northern RK Supplier). The rats were placed in plastic cages (one rat per cage) and housed in an animal room with controlled conditions involving these parameters: Temperature (22±20°C), humidity (55±10%), and lighting (12 hrs light/dark) in the animal house at the MARDI, Serdang, Selangor, Malaysia. The rats were fed with ground commercial chow and tap water (*ad libitum*). The rats were acclimated for 1 week. Their body weights were recorded weekly during a 24-week experimental period.

Chemical carcinogen

MNU (Sigma N4766-25G, Sigma-Aldrich, USA) was used to initiate the carcinogenesis. The chemical solution was freshly prepared by dissolving it in normal saline (15 mg/L).

Garlic powder preparation

Garlic cloves were obtained from a local market. They were peeled, chopped, and dried in an oven at 55°C and ground to powdered form. The garlic powder (G) was stored in refrigerator at 4°C. It was mixed with ground commercial chow and fed to the rats at 5% of feed intake [25].

Experimental design

A total of 24 8-week-old female Sprague-Dawley® rats were randomly assigned into three groups (each group consisted of eight rats), namely control, MNU, and MNU+G (Table 1). Control group rats received four consecutive SC injections of normal saline during the first 2 weeks of the experimental period (two injections per week) and fed with basal diet (ground commercial chow). MNU group rats received four consecutive SC injections of freshly prepared MNU at a dose of 60 mg/kg of body weight (total dose of 240 mg/kg of body weight) during the first 2 weeks of the experimental period (two injections per week) and fed with basal diet. MNU+G rats received four consecutive SC injections of freshly prepared MNU at a dose of 60 mg/kg of body weight (total dose of 240 mg/kg of body weight) during the first 2 weeks of the experimental period (two injections per week) and fed with diet containing 5% of garlic powder daily for 24 weeks starting at day 1 of the experimental period. The rats were euthanized after a 24-week experimental period by bleeding under general anesthesia with ketamine (at a dose of 75 mg/kg) and xylazine (at a dose of 10 mg/kg).

Methods

Histopathology

Mammary gland samples were fixed in 10% formalin for 48 hrs, processed, embedded in paraffin, and stained with Hematoxylin and Eosin (H and E) stain following the standard procedure to investigate mammary gland carcinoma lesion and MVD. MVD scoring was conducted by counting microvessels of three chosen hot spots of severe mammary gland carcinoma lesion at ×200 magnification and calculated as the average number of vessels per mm^2 [26].

Immunohistochemistry

Immunohistochemistry staining was performed on the paraffin-embedded mammary gland tissue sections (4 um) using the Dako Envision®+system/HRP, Rb (DAB+) (Dako K401011, USA) followed as per manufacturer's instruction. The VEGF protein was detected using the VEGF primary antibody (Abcam AB46154, UK). Immunohistochemistry scoring system was based on the positive expression percentage of VEGF protein and staining intensity. It was scored as 0 (<10%), 1 (10-25%), 2 (25-50%), 3 (50-75%), and 4 (>75%) for the VEGF positive expression, and 1 (weak staining), 2 (moderate staining), and 3 (strong staining) for the VEGF staining intensity. Final scores were determined by multiplying the percentage score of positive expression (P) by the intensity (I). Formula: Q = P × I [27,28].

Statistical analysis

Data obtained were statistically analyzed using the Statistical Package for Social Science (SPSS) software version 21. The statistical analysis of MVD and VEGF scoring results were performed using Kruskal–Wallis nonparametric ANOVA and Mann–Whitney for comparing between groups. The values of MVD and VEGF scoring results were expressed as mean rank. The correlation between MVD and VEGF results was analyzed using Spearman nonparametric correlation test, and the analysis of linear relationship between MVD and VEGF results was performed using linear regression test which was expressed as r^2 value.

RESULTS

Mammary gland cancer and MVD level

Ductal mammary gland carcinoma lesion was observed in rats of both MNU and MNU+G groups at different grades of severity. The lesion was characterized by massive proliferation of pleomorphic epithelial cells originated from the terminal duct-lobular unit that formed discrete clusters with duct-like morphology. These clusters of neoplastic cells had invaded the mammary gland parenchyma which abolished the mammary gland acini and causing loss of the normal mammary gland architecture (Fig. 1). Poorly structured small blood vessels were also observed and counted for the determination of MVD. The wall of these small blood vessels comprised a single line of endothelial cells.

Based on the MVD scoring results, high tumor MVD level was detected in the severe mammary gland carcinoma lesion (Fig. 1). Indeed, the new micro vessels were observed in all rats (100%) of MNU group and three rats (37.5%) of MNU+G group. The statistical analysis of MVD scoring results revealed that tumor in MVD group was significantly ($p<0.05$) higher in MNU group as compared to MNU+G and control groups, and insignificantly ($p>0.05$) different between MNU+G and control groups (Table 2).

VEGF expression

Positive expression of VEGF was observed in all rats of this study, including control rats, at different percentages and intensity levels. Overexpression of VEGF was observed in both groups of MNU and MNU+G rats. It was overexpressed in all rats of MNU group and 37.5% of MNU+G rats. The expression of VEGF protein was detected in the

Table 1: Experimental design

Group	Carcinogen	Treatment
Control	Nil	Basal diet
MNU	Four subcutaneous injections of MNU at a dose of 60 mg/kg of body weight per injection	Basal diet
MNU+G	Four subcutaneous injections of MNU at a dose of 60 mg/kg of body weight per injection	Diet containing 5% of garlic powder for a 24-week experimental period

n=8 rats. MNU: *N*-methyl-*N*-nitrosourea

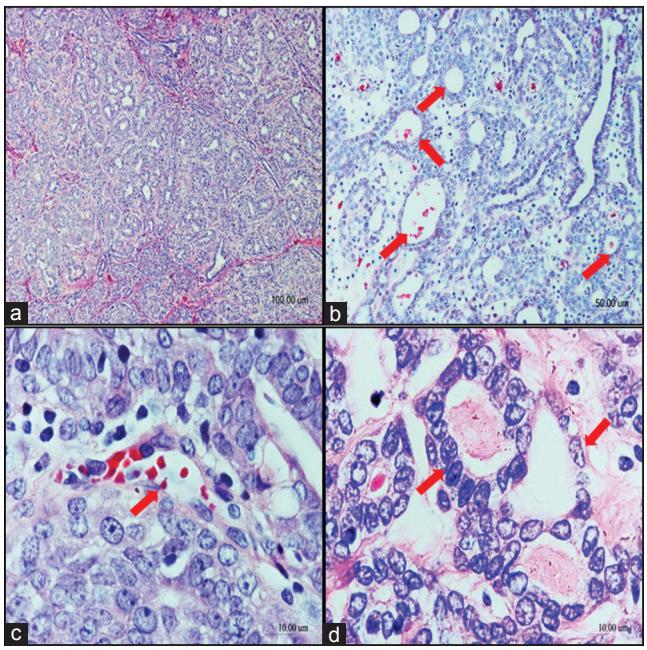

Fig. 1: Photomicrographs of mammary glands of rats exposed to *N*-methyl-*N*-nitrosourea (MNU). (a) A severe ductal mammary gland carcinoma of a MNU-exposed rat with no garlic supplementation. Note massive proliferation of neoplastic epithelial cells, derived from the terminal ductal-lobular unit, that formed discrete clusters with duct-like morphology and led to loss of the normal mammary gland architecture. (b-d) Higher magnifications of (a) showing the presence of high microvessel density of poorly structured blood vessels that are lined up with a single layer of endothelial cells (red arrows). H and E, (a) ×100, (b) ×200, (c) ×1000, and (d) ×1000

Table 2: Microvessel density scoring results of groups control, MNU, and MNU+G rats

Group	Mean rank
Control	8.38
MNU	18.69[a]
MNU+G	10.44

The letter[a] in superscript indicates the significant differences ($p<0.05$) between MNU group and control and MNU+G groups. n=8 rats each group. MNU: *N*-methyl-*N*-nitrosourea

Table 3: VEGF immunohistochemistry scoring results of groups control, MNU, and MNU+G rats

Group	Mean rank
Control	7.7
MNU	17.9[a]
MNU+G	11.3

The letter[a] in superscript indicates the significant differences ($p<0.05$) between MNU group and control and MNU+G groups. n=8 rats each group, MNU: *N*-methyl-*N*-nitrosourea, VEGF: Vascular endothelial growth factor

cytoplasm of neoplastic epithelial cells and endothelial cells of the newly formed small blood, and also at the intercellular spaces (Fig. 2).

The statistical analysis of VEGF expression scoring showed a significant ($p<0.05$) higher score in MNU group rats compared with groups MNU+G and control rats. The VEGF expression scoring showed no significant ($p>0.05$) differences between the groups MNU+G and control rats (Table 3). Correlation analysis showed that the MVD and VEGF scoring results was significantly ($p<0.01$) correlated with coefficient determination (r^2) of 0.513 (Fig. 3).

DISCUSSION

MVD is an important tool to quantify intratumor angiogenesis in cancers. High tumor MVD is always correlated with the overexpression of VEGF [29-31], which is an important growth factor in the development of cancers. Several studies have indicated the positive association between histopathological lesions of an aggressive ductal carcinoma *in situ* with high MVD and VEGF expression [29,32,33]. The findings are in accordance to the findings reported in this study where high tumor MVD and overexpression of VEGF protein were markedly observed in severe ductal mammary gland carcinoma lesion of MNU-exposed rats. The correlation analysis results further support the association between high tumor MVD and VEGF overexpression in these rats.

Many *in vivo* and *in vitro* studies reported that garlic exhibits the anti-angiogenesis activity through reducing tumor MVD and inhibiting VEGF expressions [24,34]. Similar findings were observed in this study, where administration of diet containing 5% of garlic powder significantly reduced tumor MVD and VEGF overexpression in MNU-exposed rats.

CONCLUSION

Administration of four SC injections of MNU at a dose of 60 mg/kg of body weight per injection induced the development of ductal mammary gland carcinoma in female Sprague-Dawley® rats. High levels of tumor MVD and VEGF protein expression were detected in the ductal mammary gland carcinoma with severe lesion. Daily feeding diet containing 5% of feed intake of garlic powder for 24 weeks in rats exposed to the MNU exhibited chemo preventive effects through reduction in tumor MVD and VEGF overexpression.

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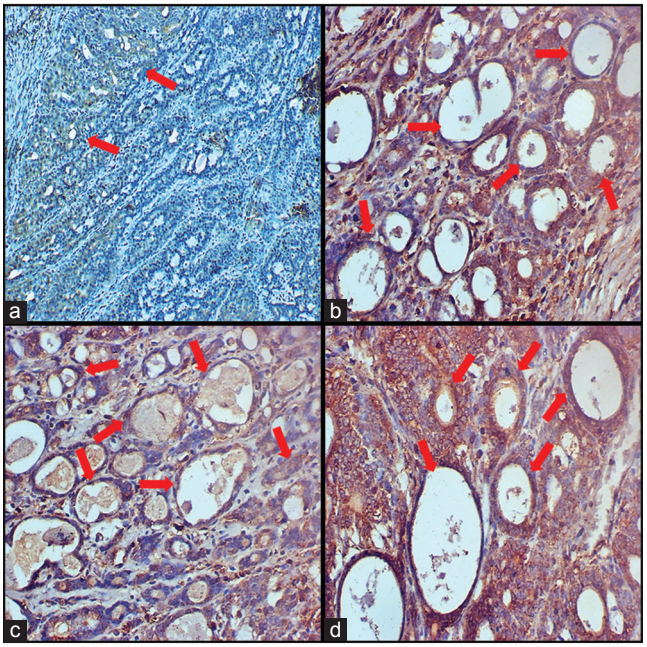

Fig. 2: Photomicrographs of immunohistochemical of vascular endothelial growth factor (VEGF) protein in mammary glands of *N*-methyl-*N*-nitrosourea (MNU)-exposed rats with and without garlic supplementation. (a) Minimal expression of VEGF protein (indicated as brownish discolouration) in a mammary gland of a MNU-exposed rat received daily garlic supplementation as a chemopreventive agent. Note the positive VEGF protein is expressed by the neoplastic cells around the newly formed blood vessels (red arrows). (b-d) MNU-exposed rats received no garlic supplementation showing higher intensity of VEGF protein expression. The VEGF protein is markedly expressed by the endothelial cells of the newly formed small blood vessels (red arrows) and also by the neoplastic cells. Note the presence of numerous newly formed small blood vessels (red arrows) indicating high microvessel density. VEGF marker and haematoxylin-IHC, (a) ×100, (b) ×200, (c) ×200 and (d) ×400

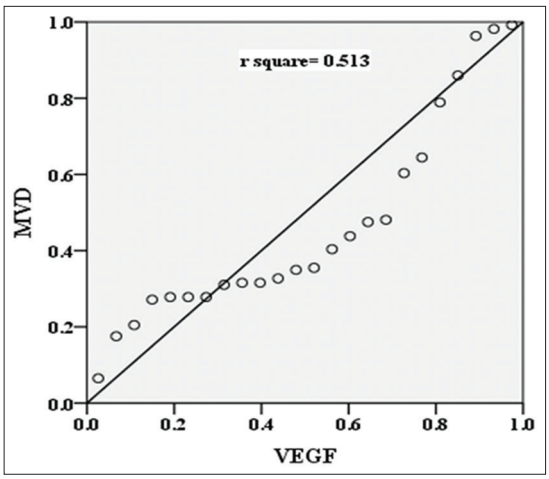

Fig. 3: Scatter plot showing positive correlation between microvessel density and vascular endothelial growth factor of groups control, *N*-methyl-*N*-nitrosourea (MNU) and MNU+G at r2 of 0.513

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