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Original Research Article

Protective Effects of Caffeine and Artemisinin against Initiation of Breast Tumour in 7, 12-Dimethylbenzanthracene treated Albino Rats

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ABSTRACT

Breast cancer is the most diagnosed cancer in women and it causes high cancer-related deaths globally. Cancer prevention strategies for breast cancer are limited hence remedies that have protective effects against the initiation of carcinogenesis are relevant. This study evaluated inhibition and effects on breast cancer by artemisinin and caffeine. Tumor development in 35 female albino rats after a dose of 40 mg/kg of 7,12-dimethylbenzanthracene (DMBA) and effects on DNA damage in breast tissues was assessed using comet assay and histology studies after 5weeks of daily administration of 25 mg/kg caffeine, 4mg/kg artemisinin or combination of both. Comet scores in treated groups were compared with positive and negative controls, at a significance level of $p < 0.05$. A mammary tumor was observed in negative control group after 54 days. Caffeine-artemisinin combination and artemisinin showed a very mild degree of DNA damage compared to the positive control. Comet values in Caffeine-artemisinin, negative control was 0.83%, 3.19%, respectively. Histopathological analyses of mammary tissues showed no evidence of neoplasm which correlated with DNA damage levels and the absence of tumor. Combination of artemisinin and caffeine may reduce the risk of developing breast cancer by inhibiting initiation, promotion and progression which is crucial for reducing cancer morbidities.

Keywords: Breast tumour, DMBA, Caffeine, Artemisinin, DNA damage.

Introduction

Global statistics on cancer is estimated at 18.1 million cases and 9.6 million deaths in 2018 and breast cancer is one of the leading cancers causing morbidity and mortality of 2,088,849 and 626,679 respectively.[1] New cases of cancer may increase by the year 2020 due to the aging population.[2] Aggressive breast cancer and higher death rates are more common in the black population.[3,4] Although, late-stage presentation as well as inaccessible diagnosis and treatment, contribute to higher cancer burdens faced by low and middle income countries,[5] therapies that aim to prevent or reduce the development and progression of cancer would be of immense benefits to reduce the overall burden of the disease. Artemisinin and caffeine are natural bioactive compounds commonly found in beverages such as coffee, Chinese tea etc, individually they possess inhibitory effects against breast and other cancer types.[5-7] Artemisinin drugs have been reported in clinical trials to show anticancer properties,[8,9] and their mechanisms of effects such as the induction of cytotoxicity, and apoptosis by activating caspase 3/7, down-regulation of anti-apoptotic proteins - survivin, and cyclin,[10] inhibition of initiation and progression by inducing growth arrest of the cell cycle at Go/G1 and G2/M check points,[11] generation of intracellular reactive oxygen species, down-regulation of TfR, Bcr/Abl protein and suppressed activity of tyrosine kinase.[9, 12-16]

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Similarly, caffeine inhibits tumor development through antagonism of the $A_{2A}R$ adenosine receptor, cell-cycle arrest downregulation of phospho-Erk1/2, phospho-Akt, survivin, and anti-apoptotic Bcl-2 protein.[17-21] Studies have also reported that caffeine improves the efficacy of anticancer drugs.[22] Despite these reports, it is not known the potential benefit of the combination of artemisinin and caffeine for cancer prevention, hence it is imperative to determine their mechanism of effects in reducing the risk of cancer and these were investigated and data reported.

Materials and Methods

This was an experimental design to determine the efficacy of artemisinin alone or combined with caffeine for chemo-prevention of breast tumor development and DNA-damage induced with 7,12-dimethylbenzanthracene (DMBA) in Wistar Albino rats. Tumors were detected by palpation around the mammary glands and once detected, the animals were sacrificed. The effects on other tissues were also evaluated. Thirty-five female rats aged between 45 and 60 days, weighing 100-150 g were purchased and housed in the Covenant University animal house. The temperature was maintained at room temperature and they were allowed to acclimatize for two weeks, and given a standard laboratory diet and water *ad libitum*. Dosing of 40 mg/kg of DMBA which is a modification of the previously reported method was administered at 2ml/kg per animal.[7] Artemisinin alone at 4mg/kg or in combination with 25 mg/kg caffeine was administered to the test group animals (Table 1) by oral gavage daily for 34 days with the use of a cannula and syringe. The dose for artemisinin and caffeine were modified based on previously reported studies on their anticancer effects.[7,23] Animals were sacrificed and breast tissues obtained was stored in 10% formalin for histology analysis, the palpable tumor was observed in the negative control group on day 54. The protocol was given by the Covenant University Health Research Ethics committee with approval number CHREC/011/2019.

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Table 1: Groups and treatment administered to *animals with* DMBA-induced tumor

Groups	Treatment		
	DMBA (mg/kg)	Artemisinin (mg/kg/day)	Caffeine (mg/kg/day)
A (5 rats)	0	0	0
B (5 rats)	40	0	0
C (5 rats)	40	0	25
D (5 rats)	40	4	0
E (5 rats)	40	4	25
F (5 rats)	40	8	25
G (5 rats)	40	4	50

Single cell gel electrophoresis (Comet assay) and Histological assessment

Agarose pre-coated slides were prepared and air-dried to have a thin film. The comet assay was performed according to an established protocol previously described[23]. Ten microlitre blood from the animal sample mixed with 75 µL of low melting point agarose (LMPA; 0.5%; at 37ºC) was placed on the coated slide, and covered with a coverslip. The slide was kept in the refrigerator overnight. The slides were lysed in cold, freshly prepared lysing solution then placed in alkaline buffer for 20 minutes. This allowed for the unwinding of the DNA and expression of alkali-labile damage. The slides were placed in electrophoresis unit and run at 24 volts (0.74 V/cm), set at 300 mA for 30 minutes. The slides were coated with neutralization buffer in drops, and allowed to sit for about 5 minutes then stained with ethidium bromide. Slides were viewed under microscopic and images were taken at 160 magnifications. For the histological method, mammary tissue was fixed in 10% formalin and embedded in paraffin. Tissue sections were prepared and stained with hematoxylin and eosin, and viewed under the microscope at magnification of x40 and x100. The percentage (%) DNA in tail, tail length, olive moment, tail moment, and comet intensity[23] were determined.

Statistical analysis

Continuous data were reported and represented as mean ± standard error of mean. The data was analyzed using the statistical software (SPSS) for descriptive statistics (version 20) and compared between groups; P value of < 0.05 was taken to indicate statistical significance. The images were taken using Olympus® microscope and comets tails were analysed with Open Comet image analysis software[24] to obtain readings of the % DNA in the tail, tail moment, tail length, olive moment and comet intensity.

Results and Discussion

Table 2 shows the comet tail showing percent DNA in animals with DMBA-induced tumors treated with caffeine plus artemisinin. Table 3 shows comet intensity in animals with DMBA-induced tumors treated with caffeine plus artemisinin. Percent DNA in the tail with a combination of caffeine and artemisinin at 25 mg/kg was 0.83277. The results showed that 25 mg/kg caffeine had a better protective activity than 4 mg/kg artemisinin with values of 2.81% and 4.17% respectively. Table 4 shows comet and histology images in the animals treated with caffeine plus artemisinin. Histological images show tissue damage in negative control compared to other groups.

The increasing incidence of cancers is alarming, globally cancer incidence and associated mortality within ten years (2008 and 2018) have been on the increase particularly in less developed countries.[1, 25] The increase in cancer incidence has been attributed to factors such as aging of the population, genetic composition, smoking, obesity, late age at first birth, poor diet, and physical inactivity[26]; some of which are inevitable. DNA damage and unrepaired DNA underlies the molecular mechanisms of the initiation and progression of cancers[27]. Comet results from our study indicate a reduced carcinogenic effect of DMBA in groups treated with artemisinin and caffeine. Under the electric current, the DNA moves out of the cell, towards the anode, like a 'comet'. The size, shape and the distribution of the DNA comet indicates the extent of single-strand breaks and double-strand break. Histology results show that there were many acini with greatly reduced lumen embedded within the high connective tissue area of the breast tissue.

Table 2: Comet tail showing percent DNA in animals with DMBA-induced tumors treated with caffeine plus artemisinin

GROUP	% DNA in tail			Mean ± SEM	P value
	Replicate 1	Replicate 2	Replicate 3		
A	2.90	3.43	8.74	5.02 ± 1.86	0.17
B	1.89	3.78	3.90	3.19 ± 0.65	0.17
C	1.33	3.19	3.90	2.81 ± 0.76	0.10
D	3.77	3.69	5.06	4.17 ± 0.44	0.02
E	0.53	0.12	2.08	0.83 ± 0.65	0.006*
F	1.17	2.67	3.46	2.43 ± 0.67	0.063^{+}
G	2.81	3.41	4.29	3.50 ± 0.43	$0.036^{\#}$

*Group E was significantly lower than group A, $^{\#}$group F and $^{+}$group G

Table 3: Comet intensity in animals with DMBA-induced tumors treated with caffeine plus artemisinin

GROUP	Comet Intensity		
	Replicate 1	Replicate 2	Replicate 3
A	43.47	18.19	85.61
B	33.94	8.72	10.49
C	16.72	15.12	25.11
D	42.72	16.17	21.75
E	19.68	16.05	16.77
F	17.97	17.72	19.62
G	37.07	43.36	13.30

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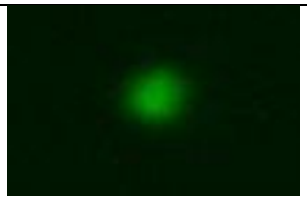
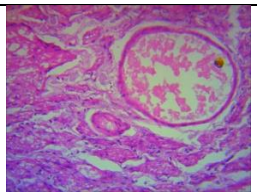
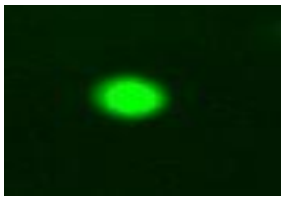
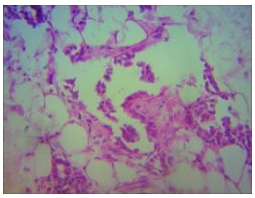
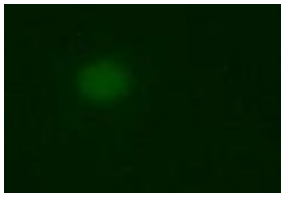
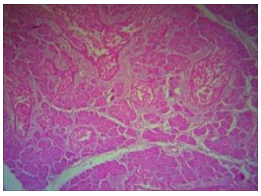
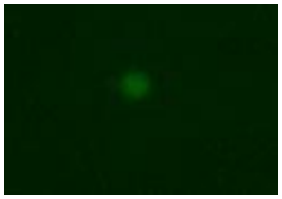
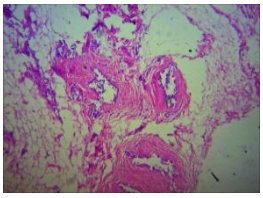
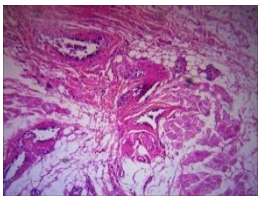
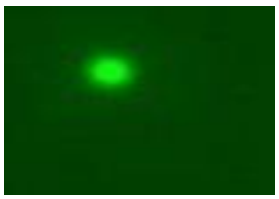
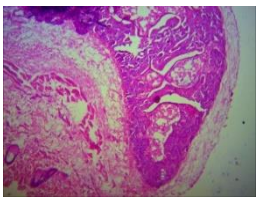
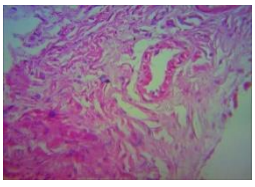
Table 4: Comet and histology micrographs for the animals during the study

GROUP	COMET IMAGE	HISTOLOGY IMAGE	COMMENT
A Negative control			Few filled glandular sections, many acini with greatly reduced lumen embedded within high connective tissue area
B DMBA positive control			No mammary acini seen
C 25 mg/kg Caf			No visible lesions seen. Well endowed/fully active mammary acini
D 4 mg/kg Art			Mammary gland connective tissue, ducts and surrounding adipose tissue seen. The glandular structures are scanty.
E 25 mg/kg Caf + 4 mg/kg Art			Interlobular ducts and connective tissue seen, but mammary lobes and acini greatly reduced and absent in many places
F 25 mg/kg Caf + 8 mg/kg Art			Ducts and connective tissue seen. The glandular structures are present in some sections, showing active secretory acini. There are large foci of marked cellular aggregation by inflammatory cells within the mammary tissue
G 50 mg/kg Caf + 4mg/kg Art			Periacinar connective tissue and ducts more prominent. Mammary secretory tissue not seen.

The high connective tissue proliferation indicates the mammary gland is either not lactating or the mammary tissue is compromised by an external factor which destroyed the tissue and laying down of scar tissue (connective tissue). Many treated animals had no tumor at 5 weeks after treatment. Review of earlier studies suggests caffeine inhibits the initiation of carcinogenic[15] and is dependent upon the dose-level and time-span of caffeine administration. There is a notable paucity of research done with caffeine on animal models of cancer[5, 14, 25]; relative to the cell line models, highlighting the importance of our findings. Caffeine has been shown to have synergistic effects with other drugs by overcoming drug-mediated cell-cycle arrest and increasing subsequent apoptosis which is buttressed by previous review studies[13] in lung cancer cells[21], in prostate cancer cells[16]. An early study provides evidence that chronic caffeine consumption can significantly inhibit the development of mammary tumors in rats treated with DMBA. Other authors investigated the early effects of caffeine on tumor initiation and progression in 3-methylcholanthrene induced tumor in animal models[14,27]. They showed that caffeine treatment increases anti-tumor immune responses by antagonising the $A_{2A}R$ (adenosine receptor) pathway. Additionally, another mechanism

by which caffeine inhibits tumor initiation in DMBA-induced tumorigenesis appears to be via an alteration in DMBA metabolism[28], by blocking mutagenic events, detoxifying the carcinogens via phase II enzymes or preventing their metabolic activation.[28] Caffeine was shown to be a blocking agent and a mediator of the chemo-preventive effects of tea on liver carcinogenesis via similar mechanism of detoxification by antioxidant enzymes. Cancer cells develop mutations in genes that regulate the cellular DNA damage response and repair pathways, tumor suppressor genes, and proto-oncogenes. This implies that cancer cells are more liable to cell cycle arrest and death from treatment with anti-cancer drugs than the normal cells of the body.

Mechanisms of anticancer effects of artemisinin includes the generation of ROS that causes oxidative stress, induction of apoptosis[7], inhibiting angiogenesis,[29]arrest of the cell cycle at G0/G1 and ferroptosis; a programmed cell death caused by lipid peroxidation [30] especially due to high intracellular iron concentration.[31] This high intracellular concentration of iron is the major target of the anticancer activity of artemisinin. Remarkably, combined administration of artemisinin (4mg/kg) and caffeine (25 mg/kg) for 34 days showed the least amount of DNA damage. The combined killing effects of pro-cancerous cell and prevention of initiation of carcinogenesis by artemisinin and caffeine respectively makes the duo effective.[28,32] This was not in a dose-dependent pattern as other groups had higher amount of damage.

Conclusion

In conclusion, this study reports effective inhibition of breast tumors with a combination of caffeine and artemisinin limiting the amount of DNA damage and hence can prevent the initiation process of breast tissue carcinogenesis.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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