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Effect of Edible Bird's Nest on Caco-2 Cell Proliferation

Aswir Abd Rashed and W.M. Wan Nazaimoon
Cardiovascular, Diabetes and Nutrition Research Centre,
Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

Abstract: The major components of Edible Bird's Nest (EBN) are glycoproteins. Amongst the carbohydrates, sialic acid (9%) is said to be most abundant in EBN. In spite of the long history of medicinal usage of EBN the actual mechanism of action remains to be elucidated. The human colonic adenocarcinoma cell line, Caco-2 cells is a widely used *in-vitro* model to study the intestinal system including the effect of different dietary components on cell growth. To assess the effect of EBN on viability and proliferation of Caco-2 cells. The EBN, supplied by the Department of Wildlife and National Parks, Kuala Lumpur comprised 2 commercial brands and 4 unprocessed samples obtained from 3 zones (North, South and East Coast) of Peninsular Malaysia. The effect of 6 EBN samples on Caco-2 cells was determined using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to EBN at concentration of 5 ppm for 24 h. The assay involved conversion of yellow tetrazolium salt MTT by mitochondrial dehydrogenases found only in metabolically active cells to an insoluble purple formazan product which was then solubilized with dimethylsulfoxide. The optical density of the homogeneous solution was read spectrophotometrically at 490 nm. The percentage of cell proliferation when treated with 2 commercial EBN, brand Y1 and brand X1 were 84 and 115% , respectively while when treated with unprocessed EBN from East Coast, North and South Zones were 91, 35 and 47%, respectively. Preliminary results showed that depending on the source and type of EBN used, there were differences in the percentage of proliferation of Caco-2 cells. Further study is needed to determine the significance of these findings.

Key words: Edible bird's nest, cell viability, caco-2 cells, cell proliferation, immune system, Malaysia

INTRODUCTION

The Edible salivary Bird's Nest (EBN) of the white-nest swiflets of *Aerodramus fuciphagus* species built during the breeding season is now becoming an important highly-priced export commodity for Malaysia. Traditionally used as food delicacy or as an important ingredient in traditional Chinese medicine, major components of EBN are mainly carbohydrates, glycoproteins and trace elements (Kathan and Weeks, 1969; Nakagawa *et al*., 2007). Sialic acid (9%) is the major component of carbohydrate in EBN (Colombo *et al*., 2003). Use of sialic acid may benefit the neurological and intellectual advantages in infants (Chau *et al*., 2003). As an excellent immune system moderator, sialic acid affects the flow resistance of mucus which in turn repels bacteria, viruses and other harmful microbes. Its related benefits include lowering of the LDL preventing influenza A and B strains increasing fertility and controlling blood coagulation. The other major carbohydrates include 7.2% N-acetylgalactosamie (galNAc), 5.3% N-acetylglucosamine (glcNAc), 16.9 galactose and 0.7% fucose (Dhawan and Kuhad, 2002). GalNAc is involved in the function of the synapses, the junction between nerve cells and deficiency can cause severe memory problems (Argueso *et al*., 2003). GlcNAc is an amino acid and a prominent precursor for glycosaminoglycans a major component of joint cartilage.

Supplemental glucosamine may help to prevent cartilage degeneration and alleviate symptoms associated with arthritis (Pasztoi *et al*., 2009). EBN has been found to potentiate mitogenic response of human peripheral blood monocytes to stimulation with proliferative agents, Concanavalin A and Phytohemagglutinin A (Yano *et al*., 2003) and was also shown to stimulate DNA synthesis in 3T3 fibroblast in a dose dependent manner, suggesting an Epidermal Growth Factor (EGF) like activity (Kong *et al*., 1987). Using EBN and pearl powder containing formulation, it was demonstrated that there was elevation of DNA synthesis of the T-lymphocytes and circulating immunoglobulin M level in mice, implying immunoenhancing effects (Zhang *et al*., 1994). The

Corresponding Author: Aswir Abd Rashed, Cardiovascular, Diabetes and Nutrition Research Centre,
Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

formulation was also shown to increase the level of superoxide dismutase. However, the study did not determine whether the effects were from EBN or pearl powder alone or the combined formulation. In spite of the long history of using EBN for medicinal purposes, there are not many scientific data to substantiate its therapeutic claims.

Due to the large increase in the demand and commercial value of EBN there is a need for more scientific evidence to substantiate the various health claims associated with its consumption. Caco-2 cell is a human colon adenocarcinoma line which upon cultivation displays numerous differentiation criteria of the small intestine epithelial cells and as such are useful and well accepted *in vitro* model for metabolism and transport studies.

The objective of this study therefore was to determine the effects of three major components of EBN, sialic acid, galNAc and glcNAc and EBN sample as a whole on the viability and proliferation of Caco-2 cells.

MATERIALS AND METHODS

Samples and preparation: The EBN samples comprised of two processed, commercial brands (Y1 and X1) and 4 unprocessed samples obtained from 3 zones (North (Zua1 and Zub1), South (ZS1) and East Coast (ZP1)) of Peninsular Malaysia were supplied by the Department of Wildlife and National Parks, Kuala Lumpur. Standard for sialic acid, galNAc and glcNAc were purchased from Sigma-Aldrich, USA.

The working concentration of commercial brand BN and unprocessed BN used was 5 ppm. All unprocessed EBN were manually cleaned of dirt and feathers allowed to dry and the grounded samples kept in air-tight containers at room temperature until use.

Pre-weighed sample was treated with 3% of 0.1 M hydrochloric acid and left overnight to dry before 1 mL of distilled deionised water was added to dissolve the sample.

Cell culture: The Caco-2 cells obtained from ATCC (HTB-37) were seeded and maintained in 25 cm^2 plastic flasks. The cells were grown in Eagle's Minimum Essential Medium (EMEM) from GIBCO, Grand Island, NY USA, supplemented with 20% v/v foetal calf serum (GIBCO), 1% v/v non-essential amino acids (GIBCO), 1% antibiotic anti-fungal (penicillin-streptomycin) solution (GIBCO) and 1% v/v L-glutamine (GIBCO). The cells were maintained at 37°C in an incubator with 5% Carbon dioxide (CO_2), 95% air atmosphere at constant 95% relative humidity and the medium replaced every 2 days.

Stimulation with commercial brand BN and unprocessed BN

Cell proliferation and viability assay: The effect of 6 EBN samples on cell proliferation and viability was determined using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded overnight at a density of 20,000 cells cm^{-2} in collagen-treated 96 well plates (Costar Corp., Cambridge, MA USA). On the next day the medium was changed to medium free FBS and EBN samples were added to the required concentrations. Again the cells were incubated for 24 h at 37°C in a humidified incubator 5% CO_2 atmosphere.

Then, 10 μL of MTT solution was added to each well including controls and incubated for 3-4 h at 37°C in a humidified incubator 5% CO_2 atmosphere. Periodically, the cells were viewed under an inverted microscope for the presence on intracellular punctuate purple precipitate. When the purple precipitate was clearly visible the media was discarded before 100 μL DMSO was added to each well including controls and swirl gently before measuring the absorbance at 490 nm. The number of surviving cells is directly proportional to the level of the formazan product created.

Statistical analysis: Data obtained were tested for significance using Analysis of Variance (ANOVA) with Duncan's multiple range test for comparing among groups using the Statistical Package for Social Sciences (SPS) version 10.0. The probability level of significance was fixed at $p<0.05$.

RESULTS AND DISCUSSION

The EBN supplied for all 6 groups were similar in their physical appearance. All unprocessed EBN were collected during the breeding season from April-July.

Effects of sialic acid, galNAc and glc NAc on cell proliferation: Stimulation with different amount of sialic acid (0, 2, 4, 6, 8 and 10) caused a dose dependant increase in cell proliferation (Fig. 1a). The first apparent effect was noted at 24 h where with 2% of sialic acid cell proliferation increased significantly by 50% ($p = 0.027$) and at 10% sialic acid, the increase >100% ($p = 0.009$) when compared with the control.

Overall optimal growth was found when growth media contained between 6-10% of sialic acid. Similar dose-dependent increase in cell proliferation was also found with galNAc (Fig. 1b) and glcNAc (Fig. 1c) with optimal growth seen at 6%. Higher amount of the carbohydrates however, markedly inhibited cell proliferation.

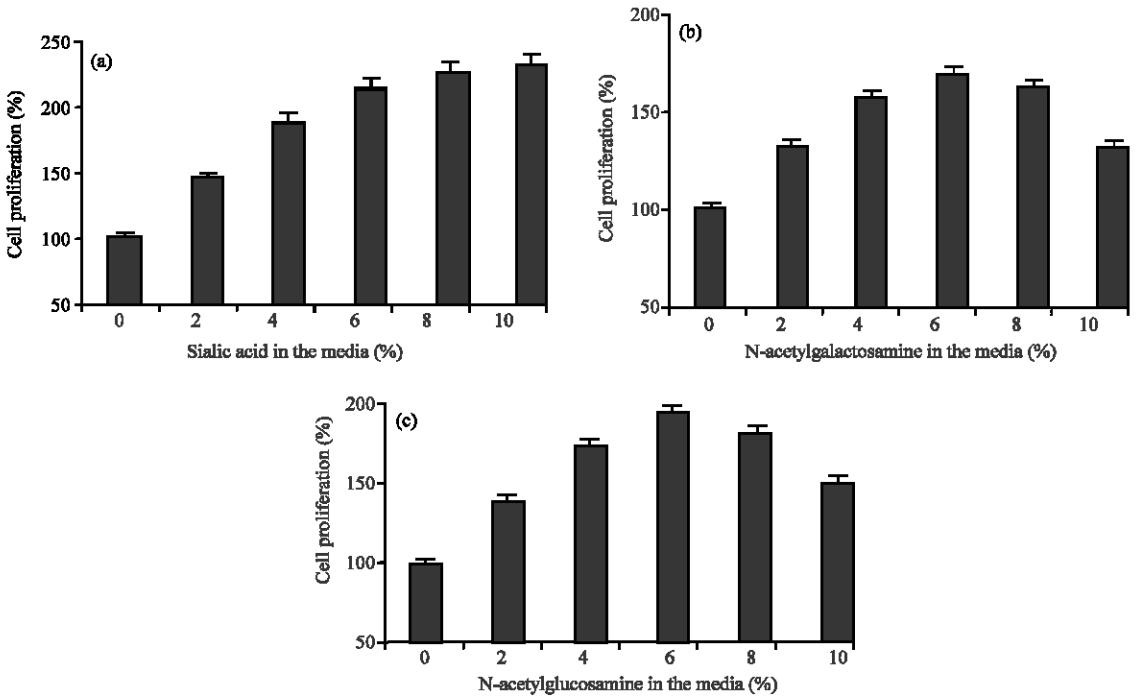

Fig. 1: Dose-response curves of Caco-2 cell proliferation produced by additions of sialic acid, N-acetylgalactosamine or N-acetylglucosamine in the serum free media

Effects of commercial and unprocessed EBN: Compared to untreated cells, there was significant cell proliferation ($p<0.05$) in cells treated with 5 ppm of either commercial or unprocessed EBN (Fig. 2). Highest percentage of proliferations were observed from cells grown in media containing commercial EBN of Brand X1 ($p = 0.01$), unprocessed EBN from East Coast ($p = 0.008$) and South Zones ($p = 0.011$), at 215±4.7, 184±7.1 and 146±3.2%, respectively.

This study used Caco-2 cells model to determine the biological effects of EBN and its major component on Caco-2 cells. To the knowledge there had no previous report on the effect of sialic acid on the proliferation of Caco-2 cells. Because sialic acid is known to exert many health-beneficial effects (Walker-Nasir *et al*., 2003), it is therefore important to verify its content especially in commercial EBN. The significant dose-dependent growth of Caco-2 cells caused by sialic acid suggests the potential use of this carbohydrate as a marker to determine its presence for checking quality and authencity of a product which may have health claims related to sialic acid. Similar principle can be applied to galNAc and glcNAc and as shown in this study excessive amount of these compounds would result in significant toxic effect on the cells. In fact, in this study Bussing *et al.* (1998) found that a much lower concentration of galNAc at 0.1 M (2.2%) was sufficient to induce apoptosis in human lymphocytes. Since there have been many health claims on EBN especially in association to its nutritional components such as sialic acid

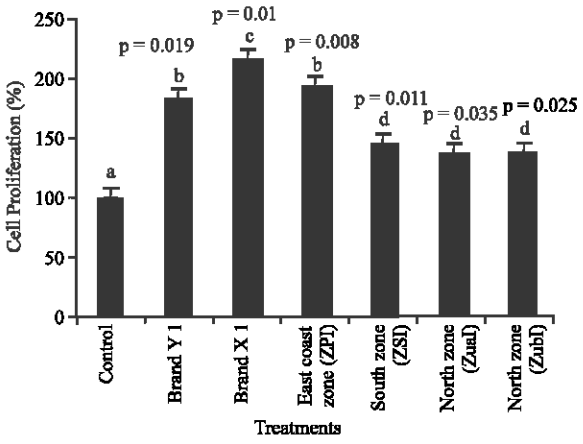

Fig. 2: Effects of EBN on Caco-2 cell proliferation. Cells were treated for 24 h with and without EBN at 5 ppm concentration in serum free media. The optical density was determined by spectophotometer at 490 nm. Values were expressed as Percentage Mean±SD of three experiments. Bar values with no common letters are significantly different ($p<0.05$)

(Walker-Nasir *et al*., 2003), galNAc (Argueso *et al*., 2003) and glcNAc (Pasztoi *et al*., 2009), there is a need to verify and substantiate such claims. It has been shown that the most common adulterants found routinely incorporated into EBN during commercial processing prior to final sale included jelly fungus, agar, sodium alginate, nature plant gum, pork skin, isinglass, egg white, karaya gum, red seaweed and tremella fungus (Tung *et al*., 2008).

In the study, Caco-2 cells grown in media containing commercial EBN Brand X1 showed the highest proliferation when compared to Brand Y1 or the unprocessed EBN, implying the presence of growth-stimulating components in this particular sample which could be either naturally present or due to adulteration. This finding needs to be further investigated and confirmed.

The differences in cell proliferation when grown in different unprocessed EBN are expected. As shown in an earlier study (Huda *et al*., 2008), there is significant differences in nutritional components of local EBN between regions and nest types.

This might be due to condition of surrounding habitats, availability and abundance of food source (Che, 2001). Studies done by several researchers (Harrisson, 1976; Langham, 1980; Hails and Amiruddin, 1981) found that swiflets feed on diverse array of aerial arthropods with similar sized birds taking a similar range of prey sizes. Lourie and Tompkins (1999) on Malaysian swiflets found that swiflets diets are very selective where hymenoptera (medium to large inserts with two pair of wings) and diptera (small inserts with a single pair of wings) were the most abundant prey in all diets. For the habitat comparisons diptera were the main constituents of the swiflets diet in an urban habitat whereas hymenoptera predominated in forest habitat (Lourie and Tompkins, 1999).

CONCLUSION

In conclusion, the results presented in this study demonstrated that there is a need and important to monitor and verify the various health claims made on commercial EBN especially its nutritive contents, not only for consumer protection but also to ensure that the product is safe for human consumption.

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