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Effect of edible bird's nest on cell proliferation and tumor necrosis factor- alpha (TNF-α) release *in vitro*

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Abstract: The human colonic adenocarcinoma cell line (Caco-2 cells) and a macrophage cell line (RAW 264.7 cells) are widely used *in vitro* model to study the intestinal system and Tumor Necrosis Factor- alpha (TNF-α) release in cells, respectively. The objectives of this study were to assess the proliferative effect of EBN on Caco-2 cells and its effect on TNF-α release in RAW cells. 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. Several EBN from specific zones (brand Y1, South and East Coast Zones) significantly affect the TNF-α production in RAW cells, where it was reduced to 43%, 24% and 32% respectively. Results showed that depending on the source and type of EBN used, there were differences in the percentages of proliferation of Caco-2 cells. EBN is able to influence the production of anti-inflammatory TNF-α in RAW cells.

Keywords: Edible bird's nest, cell viability, cell count, caco-2 cells, RAW 264.7 cells

Introduction

There are approximately 200 natural sugar compounds, but only 8 are essential to bodily function. The 8 essential sugars are known as glyconutrients and required for proper bodily functions. There are xylose, fucose, galactose, glucose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid (sialic acid). Seven of the eight glyconutrients are found in edible bird's nest (EBN). This gives us an indication how valuable EBN are. There are several subspecies of the edible-nest swiftlets namely fuciphagus, maximus, inexpectatus, dammermani, micans, vestitus and perplexus. Since 16th century, EBN soup became a delicacy in Chinese cuisine as well as an important part of alternative medicine (Medway, 1969) and as a beauty treatment because of its skin rejuvenation properties and its Epidermal Growth Factor (EGF) that is responsible for skin and tissue repair (Kong *et al*., 1987). Nowadays, the EBN of *Aerodramus fuciphagus* believed to provide health benefits, forming the basis of a multi-billion dollar industry worldwide.

The composition of EBN from genus Aerodramus includes lipid (0.14–1.28%), ash (2.1%), carbohydrate (25.62–27.26%) and protein (62–63%)(Marcone *et al*., 2005). One of the major glyconutrients in EBN is sialic acid 9% (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 low-density lipoprotein (LDL), preventing influenza A & B strains, increasing fertility and controlling blood coagulation. The other major glyconutrients include 7.2% N-acetylgalactosamine (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 (Argüeso *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). Galactose and fucose are glyconutrients that have an impact on brain development, proper cellular communications and display antibacterial properties.

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.

To determine the effects of EBN on cell proliferation, we used human colonic adenocarcinoma

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cell line (Caco-2 cells) because it is a well accepted *in vitro* model for metabolism and transport studies. To assess whether EBN reduces inflammation, we investigated its anti-inflammatory properties in mouse leukaemic monocyte macrophage cell line (RAW 264.7). This cell line is a widely used model for studying signal transduction pathways.

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

The working concentration of commercial brand EBN and unprocessed EBN 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.

The diluted EBN was prepared by a simple acid digestion technique as per procedure suggested by Wang (1921), with slight modification. In brief, the pre-weighed samples were treated with 3% of 0.1M hydrochloric acid and heated for an hour at 80ºC to dissolve the EBN samples. The pH of the sample was adjusted to 7.0 with 0.1N sodium hydroxide.

High performance liquid chromatography (HPLC) was used to determine the concentration of sialic acid, galNAc and glcNAc in the respective EBN samples (data not shown) (Norhayati *et al*. 2010) and to decide the concentration of glyconutrients should be applied for later experiments.

Standard for sialic acid, galNAc and glcNAc were purchased from Sigma-Aldrich, USA.

Cell lines and culture conditions

The Caco-2 cells, obtained from the America Type Culture Collection (ATCC), were seeded and maintained in 25 cm^2 plastic flasks (Costar, Cambridge, MA). 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 37^0C in an incubator with 5% carbon dioxide (CO_2), 95% air atmosphere at constant 95% relative humidity, and the medium replaced every 2 days.

The RAW 264.7 cells were propagated in 25 cm^2 plastic flask (Costar, Cambridge, MA) in Dubelco's Modified Essential Medium (DMEM) 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 37^0C in a humidified atmosphere containing 5% carbon dioxide (CO_2), Before use, cells were detached at 37°C with trypsin-EDTA (GIBCO) and plated in 96-well microtiter plates at a density of 200,000 cells/200-µl well. The medium was replaced every day, and cells were used after 48-72 h of incubation.

Evaluation of 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. MTT reagent was purchased from ATCC. The cells were seeded overnight at a density of 20,000 cells/cm^2 in collagen-treated 96-well plates (Costar, 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 hours at 37^0C 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 hours at 37^0C 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 dimetyl Sulphoxide (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.

ELISA technique for TNF-α assays

TNF-α assays were measured in aliquots of culture supernatants (RAW 264.7 cells) in 96-well plates (Costar, Cambridge, MA). Samples or standards were added at 100 µl/well, and 50 µl/well biotylinated anti-TNF-α (BenderMedsystem, Austria) was added to all wells and incubated for 3 h at room temperature. The standard curve measured the range that our studies covered in between 50 and 1,000 pg/ml. The plate was then washed three times, after

which 100 µl of streptavidin-horse radish peroxidase (HRP) (BenderMedsystem, Austria) was added to all wells. The plate was incubated for 30 min at room temperature and then washed three times. The chromogen solution of transmembrane (TMB) was added at 100 µl/well and the plate was incubated at room temperature in the dark for 15 min. The chromogenic reaction was stopped by the addition of 100 µl/well of 0.2 M H_2SO_4. The optical densities (OD) were measured using a plate reader (BioRad, CA, USA) at 450 nm. A linear standard curve was generated by plotting the average absorbance on the vertical axis versus the corresponding TNF-α standard concentration on the horizontal axis. The amount of TNF-α in each sample was determined by extrapolating OD values to TNF-α concentrations in the standard curve.

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 (SPSS) version 10.0. The probability level of significance was fixed at $p<0.05$.

Results

Effects of sialic acid, galNAc and glcNAc on cell proliferation

Figure 1 shows that stimulation with different amount of sialic acid (0, 2, 4, 6, 8 and 10) caused a dose dependant increase in cell proliferation. The first apparent effect was noted at 24h where with 2% of sialic acid, cell proliferation increased significantly by 50% ($p=0.027$) and at 10% sialic acid, the increase more than 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 and glcNAc with optimal growth seen at 6%. However, higher amount of glyconutrients markedly inhibited cell proliferation (Figure 1).

Effects of commercial and unprocessed EBN on cell proliferation

Compared to untreated cells, there was significant cell proliferation ($p<0.05$) in cells treated with 5ppm of either commercial or unprocessed EBN (Table 1). The highest percentage of cell proliferation was observed from cells grown in media containing commercial EBN of Brand X1 at 215.07 ± 4.74%

Table 1. Effects of EBN on Caco-2 cell proliferation

Type of treatments	Cell Proliferation (%)
Control	100.00 ± 8.00[a]
Brand Y1	183.94 ± 7.11[b]
Brand X1	215.07 ± 4.74[c]
East Coast Zone	190.98 ± 3.61[b]
South Zone	146.33 ± 3.66[d]
North Zone (a)	135.48 ± 10.50[d]
North Zone (b)	137.53 ± 7.81[d]

Cells were treated for 24 hours with and without EBN at 5ppm concentration in serum free media. The optical density was determined by spectophotometer at 490 nm. Values were expressed as Percentage Mean ± Std of three experiments. Mean values with no common letters (a,b,c and d) are significantly different ($p<0.05$).

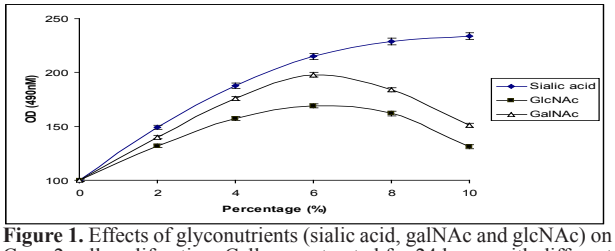

Figure 1. Effects of glyconutrients (sialic acid, galNAc and glcNAc) on Caco-2 cell proliferation. Cells were treated for 24 hours with different percentage of glyconutrients in serum free media. The optical density was determined by spectophotometer at 490 nm. Values were expressed as Percentage Mean ± Std of three experiments.

TNF-α assay on RAW 264.7 cells

Table 2 presents the production of TNF-α by RAW 264.7 cells after incubation with EBN at 5ppm concentration. Percentage of TNF-α production was reduced to 43% (430 pg/ml) when Brand Y1 was added into the media compared with the control group (800 pg/ml). The reduction effect was also occurred when EBN from other zones mainly East Zone and South Zone was added into the media at 31% (310 pg/ml) and 24% (240 pg/ml), respectively.

When broken down by individual treatments, the production of TNF-α was intriguing. GlcNA was found to be the most effective tested glyconutrient to reduce TNF-α production. The reduction was apparent at 2ppm concentration of GlcNAc. It can be seen that when low levels of glyconutrients employed into the media, the TNF-α production was reduced. However, higher concentration of glyconutrients presented into the media slowly returned the production of TNF-α back to the basal level. The obtained values of reduction efficiency on the production of TNF-α are presented in Figure 2.

Table 2. Effects of EBN on TNF-α production in unstimulated RAW 264.7 cells

Type of treatments	TNF-α production (pg/ml)
Control	805.61 ± 48.34[a]
Brand Y1	460.05 ± 61.29[b]
Brand X1	734.39 ± 10.94[c]
East Coast Zone	555.73 ± 7.05[b]
South Zone	615.74 ± 42.97[b]
North Zone (a)	1035.78 ± 44.88[d]
North Zone (b)	924.74 ± 80.28[d]

Cells were treated for 24 hours with and without EBN at 5 ppm concentration in serum free media. The biotylinated anti-TNF-α was added into the well containing the aliquots of culture supernatants and incubated for 3 h. Later, streptavidin-HRP and chromogen solution were added and incubated in the dark for 15 min before H_2SO_4 was added to stop the reaction. The OD was measured at 450 nm. Values were expressed as Percentage Mean ± Std of three experiments. Mean values with no common letters (a,b,c and d) are significantly different ($p<0.05$).

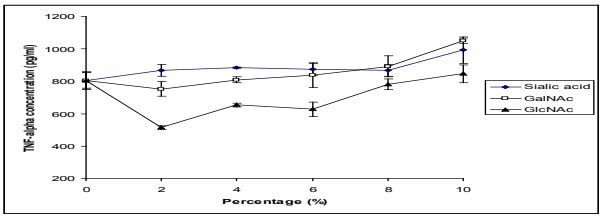

Figure 2. Effects of selected glyconutrients (sialic acid, galNAc and glcNAc) on TNF-α production in unstimulated RAW 264.7 cells. Cells were treated for 24 hours with different percentage of glyconutrients in serum free media. The biotylinated anti-TNF-α was added into the well containing the aliquots of culture supernatants and incubated for 3 h. Later, streptavidin-HRP and chromogen solution were added and incubated in the dark for 15 min before H_2SO_4 was added to stop the reaction. The OD was measured at 450 nm. Values were expressed as Percentage Mean ± Std of three experiments.

Discussion

Evaluation of cell proliferation *in vitro* is essential for toxicology and carcinogenesis studies and also to assess the efficiency of nutrient absorption in nutrition research (Melnick *et al*., 2008). Thus, we investigated the effect of selected important glyconutrients on Caco-2 cell proliferation in order to better understand the cell response in their present. We used Caco-2 cells as a host cell to try to mimic characteristics of human intestinal system.

By introducing sialic acid, we found that there was a 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, and for checking quality and authenticity of a product which may have health claims related to sialic acid (Figure 1). A similar principle can be applied to galNAc and glcNAc, as shown in this study. Tung *et al*., (2008) reported that sialic acid content provides a basis to estimate the content of authentic EBN in sample. In addition, the content ratio of sialic acid to galNAc can serve as an indicator for identifying the quality of sample claimed as EBN. Nevertheless, our study showed that their benefit functions has a certain limit, except for sialic acid, an excessive amount of galNAc and glcNAc present in the media containing Caco-2 cells resulted in a slower cell proliferation and may possibly became toxic to the cells (Figure 1).

In addition, we believed that cell proliferation have an impact on colonic mucin synthesis in Caco-2 cells. Sialic acid, galNAc dan glcNAc are part of the important components of mucin, the backbone of the intestinal tract's protective cover (Macfarlane *et al*., 2005). Van Klinken and his co-workers (1996) found that Caco-2 cells were capable to produce mucin (MUC1, MUC3, MUC4 and MUC5) at high levels. Although Niv *et al*., 1992 found that the spontaneous post-confluent enterocytic differentiation in Caco-2 cells with increased brush-border enzyme expression was associated with a decrease in mucin synthesis, adding specific glyconutrients such as sialic acid, galNAc and glcNAC into the media may have a distinctive effect in mucin synthesis. However, further investigations are needed to confirm the presence of interaction between cell proliferation and mucin synthesis in Caco-2 cells.

We also observed increased cell proliferation in response to treatment with unprocessed and commercial EBN available in the Malaysian market (Table 1). The EBN, which is claimed to be rich in glyconutrients strongly increased Caco-2 cell proliferation. The present study is the first, to our knowledge, to demonstrate that EBN increases cell proliferation by using Caco-2 cell model system. The Brand X1 showed the highest percentage of cell proliferation than the other groups, which was determined by MTT assay at 215.07±4.74%. The North Zone's EBN showed the least proliferation effect on Caco-2 cells at only 135.45±10.50%. There are several reasons for this, including the present of adulterants incorporated into EBN (Marcone, 2005; Tung *et al*., 2008), and differences in nutrition components of EBN between regions and nest types (Nurul Huda *et al*., 2008).

Since there are many health benefit claims of EBN, we decided to determine the anti-inflammatory activity by using colorimetric ELISA kit. Autoimmune disorders such as diabetes, rheumatoid arthritis and inflammatory bowel disease have all been linked to increased levels of TNF-α. In this study, we demonstrated the effect of EBN on TNF-α production in unstimulated RAW 264.7 cells. The experiments demonstrated that even without LPS stimulation, some samples of EBN were significantly effective to decrease the TNF-α production ($p<0.05$) particularly Brand Y1, East Coast Zone and South Zone at 42.86%, 30.93% and 23.6%, respectively (Table 2). GalNAc found to be the most effective tested glyconutrients to reduce the TNF-α production ($p<0.05$). Nevertheless, the percentage amount employed into the media need to be lower than 8% (Figure 2).

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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