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EFFECT OF BLACK RASPBERRY ON NF-κB AND CASPASE-3 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA VERSUS KERATINOCYTES AND FIBROBLASTS CELL LINES

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ABSTRACT

Review: Oral squamous cell carcinoma (OSCC), the most common type of intraoral cancers, is an important health issue. Most cytotoxic drugs have side effects on normal tissues beside being cytotoxic to malignant cells. Many studies demonstrated the ability of black raspberries (BRBs) to inhibit many types of cancer. However, understanding how the bioactive compounds in BRBs drive the metabolic and molecular pathways that lead to oral cancer chemoprevention remains unclear.

Aim of study: The current study aimed to investigate the consequences of BRBs treatment on (NF-κB) and caspase-3 expression in two types of head and neck SCC cell lines in comparison to both normal keratinocyte and fibroblast cell lines.

Results: There was an inhibitory effect of BRBs on both tested malignant cell lines (SCC9), and (HEp-2) and at the same time there was no effect on growth of the tested normal cell lines (HaCaT) and (BJ). There was a statistically significant increase in median fold change of caspase-3 in the tested malignant cell lines and a statistically significant decrease in the median fold change of NF-κB in the tested malignant cells with no detectable change for both antigens in the normal cell lines.

Conclusion: The results of the present study provide more evidence that BRBs have a very promising cytotoxic effect on oral cancer cell lines with no detectable harmful effect on normal cells.

KEY WARDS: Squamous cell carcinoma cell line, Caspase-3, NF-κB, Black raspberry.

INTRODUCTION

Oral squamous cell carcinoma (OSCC), the most common type of intraoral cancers, is an important health issue. OSCC is a serious locally invading tumour, which may invade the soft tissue and bone, and can spread to nerves, lymphatics and blood vessels. So it can spread and give rise to cervical lymph nodes metastasis and distant metastasis. Moreover, regardless the great attempts to upgrade

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anti-cancer drugs, the cure rates for late-stage oral SCC remain frustrating. [1,2,3]

Most cytotoxic drugs have side effects on normal tissues beside being cytotoxic to malignant cells. Destruction of proliferating normal cells is considered one of the obstacles that limits chemotherapy of cancer. For example among the most important cytotoxic drugs is doxorubicin which shows severe cytotoxic effect against human normal oral keratinocytes, also 5-Flourouracil drug stimulates ROS production which give rise to oral mucositis. So the last few decades showed increased attention on the natural herbs or plants, because of their limited complications and little side effects compared to ordinary chemotherapy.[4,5,6,7]

Many studies demonstrated the ability of black raspberries (BRBs) to inhibit many types of cancer. However, understanding how the bioactive compounds in BRBs drive the metabolic and molecular pathways that lead to oral cancer chemoprevention remains unclear.[8]

The bioactive phytochemicals in BRBs are classified according to structure and chemical category into phenolic acids, flavonoids (anthocyanins, flavanols), condensed tannins (proanthocyanins), hydrolyzable tannins (ellagitannins and gallotannins), stilbenoids, lignans, , sterols.β-sitosterol, ferulic acid and quercitin. The antioxidant effect of BRBs is caused mainly by anthocyanins and ellagitannins fractions. [9,10]

Besides the previous components, BRBs contain many other constituents known to exert cancer-protective effect like vitamins A, C, E, folic acid, calcium and zinc. The anti-cancer effect of these constituents is attributed to their antioxidant power.[11]

BRBs bioactives have several functions in cancer protection not only treatment. Among these functions, the defence against DNA deterioration by the elimination of reactive oxygen species, suppression of DNA damage induced by chemical carcinogens and alterations of expression of cellular molecules involved in cellular growth, angiogenesis and programmed cell death.[12]

Abnormal apoptosis has a major role in the development of malignancy. Evasion from apoptosis might be one of the main mechanisms by which malignant tumours fight the usual chemotherapeutic protocols.[13]

Apoptosis is stimulated by activation of caspases protein family through two different pathways known as intrinsic and extrinsic pathways. Caspases are classified into: initiator caspases (caspase-2, -8, -9 and -10) and effector caspases (caspase-3, -6 and 7), they act on many cellular proteins , finally ending up with cell death. Because of the low amount of tissue destruction associated with apoptosis, it is considered one of the main strategies of anti-cancer treatment.[14,15,16]

The transcription factor nuclear factor- ϰB (NF-κB) regulates the expression of different genes which are implicated in immune and inflammatory reactions, proliferation, cancer development and cell survival. Several studies proved that NF-κB signalling pathway contributes to cancer development and it is responsible for increased invasion, survival, chemoresistance, and angiogenesis in a number of cancer types, including OSCC . As a result, NF-κB is considered as an influential anti malignant target.[17,18,19,20]

To our knowledge a few studies have been conducted to investigate the possible effect of (BRBs) on both oral malignant and normal cells. So the current study aimed to investigate consequences of BRBs treatment on (NF-κB) and caspase-3 expression in two types of head and neck SCC cell lines in comparison to both normal keratinocyte and fibroblast cell lines.

MATERIALS AND METHODS

Cell lines: SCC 9, HEp-2, HaCaT (keratinocytes) and BJ (fibroblasts) cell lines of the American Type Culture Collection (ATCC)® were obtained from VACSERA-EGYPT. The cells were grown in DMEM containing 10% fetal bovine serum, 10 ug/ml of insulin and 1% penicillin-streptomycin at 37°C. All of the chemicals and reagents were from Invitrogen.

Black raspberry: Freezed dried whole black raspberry extract powder was purchased from (BerriHealth, Corvallis, OR, USA) ®.

Cell Proliferation Assay (MTT Assay)

The viability of the tested cell lines was established using the MTT (3-[4,5-dimethylthiazol2-yl]-2.5-diphenyl tetrazolium bromide) in vitro toxicology assay kit, MTT based, Stock No. TOX1 (Trevigen SIGMA, Saint Louis, Missouri, USA) as per manufacturer's recommendations. In brief, cells were added in a 96-well tissue culture plates in a range of 103–105 cells/well in a 100uL of the culture medium then were left overnight. The MTT reagent was applied (10 ul / well) then the cells were incubated for 12 h to promote the formation of insoluble purple formazan dye. The formazan dye was dissolved before evaluation of the absorbance of each cell line in a ROBONIK P2000 Spectrophotometer, wave length:450-560 nm. Using the MTT assay, the IC50 (Inhibition Cellular Proliferation by 50%) of black raspberry for each group was calculated. The RT-PCR thereafter were performed using the calculated IC50 for 48 hours duration for each cell line.

Real time PCR

To determine the mRNA levels of Caspase-3 and NF-ϰB in all tested groups, one-step qRTPCR test was done. The RNA isolation was performed with RNAiso reagent. Onestep qRT-PCR was carried out with Qiagen One Step SYBR® PrimeScript TM PLUS RT-PCR Kit on StepOne real time PCR machine by ΔΔCt method and mRNA level of actin was utilized as endogenous control.

The primers applied to define the mRNA level of Caspase-3 and NF-ϰB were purchased from Invitrogen : The base sequences of the two genes were as follows

Caspase-3: CASP3-F 5'-TTC ATT ATT CAG GCC TGC CGA GG-3'

CASP3-R 5'-TTC TGA CAG GCC ATG TCA TCC TCA-3'

NF-κB: NF-ϰB F 5'- ATGGCTTCTATGAGGCT-GAG -3'

NF-κB R, 5'- GTTGTTGTTGGTCTGGATGC -3'

β-actin: β-actin F 5'-GTGACATCCA-CACCCAGAGG-3'

β-actin R 5'-ACAGGATGTCAAAACTGCCC-3'

Statistical analysis

The results were tabulated using Microsoft Excel (Microsoft Office 2007). Every group was included. The results were assessed using the Statistical Package for Social Science (SPSS 15.0) Software. Data were represented as median, range, mean and standard deviation (SD) values. Kruskal-Wallis test was used to compare between the four groups. Dunn's test was applied for pair-wise comparisons when Kruskal-Wallis test is significant. Results were considered significant when P value ≤ 0.05.

RESULTS

Cell Proliferation Assay (MTT Assay)

MTT assay results showed that the mean IC 50 value in all different tested cell lines (SCC9), (HEp-2), (HaCaT) and (BJ) was 14.6, 18.2 ,105.1 and 144.4 ug/ml respectively. The (BJ) cells showed the statistically significant highest median IC50 level. Followed by (HaCat) cells which showed the

second highest IC 50 value. Both (SCC9 and HEp-2) cell lines revealed the statistical significant lowest median IC50 levels with no statistical significant difference between them. (Table 1, fig 1)

TABLE (1): Descriptive statistics and results of Kruskal-Wallis test for comparison between IC50 levels in the different groups

Group	Median	Range	Mean	SD	*P*-value
SCC9	14.6 [C]	14 – 15.4	14.67	0.7	0.016*
HEp-2	18.2 [C]	17.5 – 19.1	18.2	0.82	
HaCaT	105.13 [B]	100 – 110.4	105.13	5.2	
BJ	145 [A]	138 – 150.4	144.47	6.22	

Significant at P ≤ 0.05

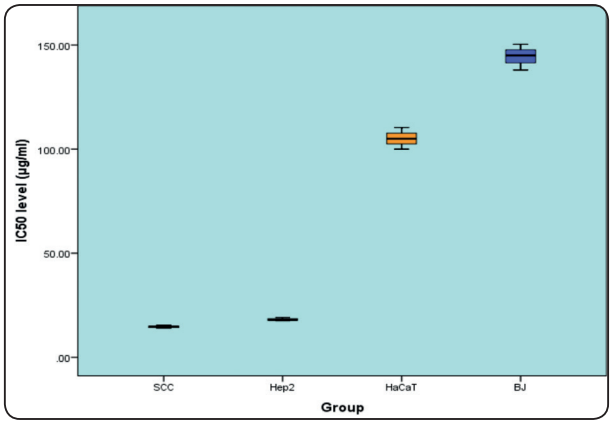

Fig. (1): Box plot representing median and range values for IC50 levels in the different tested groups

difference between them, while (HaCaT and BJ) cell lines showed the lowest statistical significant median fold change with also no significant difference between them. (Table 2,fig 2)

TABLE (2): Descriptive statistics and results of Kruskal-Wallis test for comparison between Caspase-3 fold changes in the different groups

Group	Median	Range	Mean	SD	P-value
SCC9	6.73 [A]	6.53 – 8.97	7.41	1.35	0.025*
HEp-2	5.45 [A]	3.09 – 5.69	4.74	1.44	
HaCaT	1.18 [B]	1.15 – 1.33	1.22	0.1	
BJ	1.18 [B]	1.12 – 1.31	1.2	0.1	

**: Significant at P ≤ 0.05*

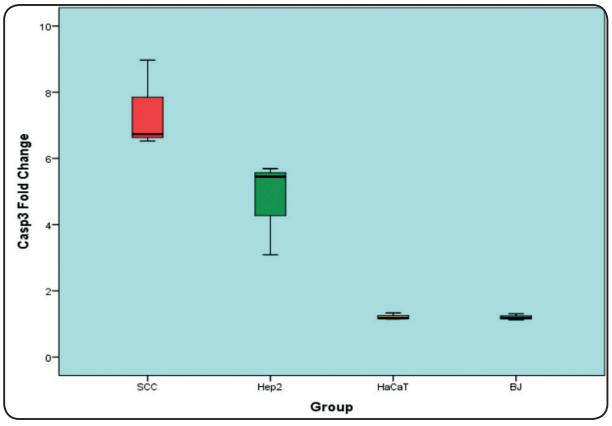

Fig. (2): Box plot representing median and range values for Caspase-3 fold change in the different tested groups

Real Time PCR

The effect of BRBs on the expression of caspase-3 in all tested cell lines (SCC9), (HEp-2), (HaCaT) and (BJ) is shown in (table 2), the highest significant expression of median fold change of caspase-3 was observed in (SCC9) cells followed by (HEp-2) cells, with no statistical significant

The effect of BRBs on the expression of NF-κB in all tested cell lines (SCC9), (HEp-2), (HaCaT) and (BJ) is shown in (table 3), the lowest significant expression of NF- κB was observed in (SCC9) cells followed by (HEp-2) cell line with no statistical significant difference between them. While (HaCaT and BJ) cell lines showed the

statistical significant highest median fold change with also no significant difference between them. (table 3, fig 3)

TABLE (3): Descriptive statistics and results of Kruskal-Wallis test for comparison between NF-κB fold changes in the different groups

Group	Median	Range	Mean	SD	P-value
SCC9	0.17 [B]	0.12 – 0.19	0.16	0.04	0.025*
HEp-2	0.17 [B]	0.15 – 0.21	0.17	0.03	
HaCaT	1.03 [A]	0.99 – 1.07	1.03	0.04	
BJ	0.91 [A]	0.83 – 0.96	0.9	0.07	

**: Significant at P ≤ 0.05*

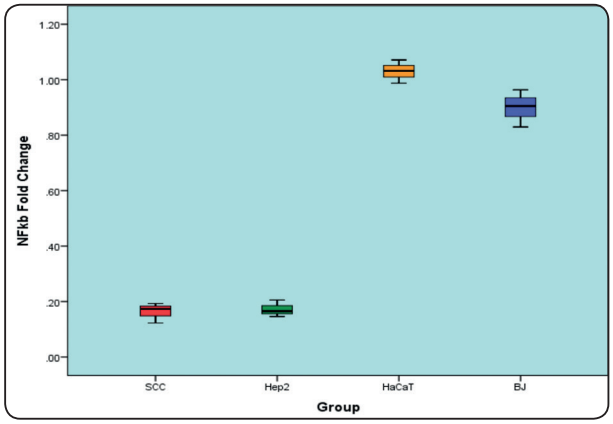

Fig. (3): Box plot representing median and range values for NF-κB fold change in the different tested groups

DISCUSSION

Many studies revealed that eating much fruits and vegetables reduces the occurrence of cancer. Fruits and vegetables are considered as a main sources of antioxidants, which hugely fight cancer. [9,12,21]

Black raspberry is one of the most used types of berries in markets. Between the dominant antioxidant constituent in berries are the anthocyanins and ellagitannins, which play a major role in its anti-cancer effect.[12,22]

Some researchers have examined the BRBs different effects on cancer, such as inhibition of tumour proliferation, angiogenesis and promotion of programmed cell. [12,23] For instance BRBs extracts has been shown to reduce the proliferation of human cervical , oral ,breast, colon and prostate cancer cell lines.[24,25]

In this study, we have broadened our recognition of the mechanism of action of BRBs ,by comparing its effect on proliferation and apoptosis on both malignant oral cell lines and normal cells lines.

In the current study, MTT assay revealed inhibitory effect of BRBs on both tested malignant cell lines (SCC9) and (HEp-2), with IC 50 value 14.6, 18.2 ug/ml respectively and at the same time there was no effect on growth of the tested normal cell lines (HaCaT) and (BJ), where the 48-hr neoplastic IC50 dosages were not toxic to the normal cells and the normal cells showed inhibition of proliferation at a very high values of IC50. **Han C. et al, 2005,** have demonstrated that black raspberries prevent the proliferation of only premalignant and malignant but not normal human cell lines.[26,27]This result is in agreement with our study results that showed the selective inhibition of BRBs on the growth of only cancer cell lines.

Programmed cell death is controlled by caspases family, which induces the release of cytochrome c and activates many damaging enzymes that digest many vital cellular proteins .[28]

Apoptosis is considered one of the main strategies of anti-cancer treatment. So in the present study the effect of BRBs on apoptosis in OSCC was examined. Our results revealed that BRBs extract induced the over expression of caspase-3 in the tested malignant cell lines (SCC9) and (HEp-2) where both showed the highest mean values. While the normal cell lines (HaCaT) and

(BJ) both showed the lowest statistical significant mean value of caspase-3. This result was in agreement with other previous studies, where BRBs induced selective activation of apoptosis in only highly malignant cells, through stimulation of caspases-3 and 7.[29,30]

The antiapoptotic effect of BRBs may be due to the influence of anthocyanin and Quercetin, through the induction of cell cycle arrest while going through apoptosis with the inhibition of Bcl-2, and the stimulation of the Bak, Bax, cytochrome c and caspase-3 expression.[31]

NF-κB is significantly increased in OSCC and its overexpression induces epithelium–mesenchyme transition (EMT) and increases the expression of degradation enzymes, such as matrix metalloproteinase (MMP)-9 indicating that NF-κB may significantly allow progression and metastasis in OSCC.[20,32,33]

The present study revealed that BRBs extract induced the inhibition of NF-κB expression in the tested malignant cell lines (SCC 9) and (HEp-2), where both showed the statistically significant lowest median fold change compared to the control. While the tested normal cell lines showed the statistically significant highest median fold change. Our results were in accordance with previous studies, where BRBs inhibited the NF-κB expression in breast cell line (MCF-7) and mouse epidermal cells that were transfected with (NF-κB). Some previous studies mentioned that anthocyanins may be responsible for the inhibition of NF-κB activity through the prevention of mitogen-activated protein kinase activation and the phosphorylation of the inhibitory subunit κB.[12,34,35]

Its worth mentioning here that, the principle for this selectivity of BRBs on only tumour rather than normal cells may be explained by **Zikiri et al,2009,** findings who clarified that this selectivity may be attributed, partially, to the biased uptake of anthocyanin by only malignant tumours. The mechanism of the preferential uptake and retention of black raspberry anthocyanin is still obscure, but may be linked to the fact that cancer cells are more energetic in pinocytosis state so they could "ingest" extracellular black raspberry more efficiently. Also this selective uptake in particular cells may be a receptor-mediated process. If specific type of cells has more receptors for anthocyanin then other, they will be able to retain more amount of black raspberry anthocyanin by receptor-mediated endocytosis.[29]

CONCLUSION

The results of the present study provide more evidence that BRBs have a very promising selective cytotoxic effect on oral cancer cell lines with no detectable harmful effect on normal cells, this cytotoxic effect was confirmed by the inhibition of NF-κB pathway and the activation of caspase-3.

RECOMMENDATIONS

Future investigations is needed to study the effect of each individual constituent of BRBs on OSCC to find out how strong the effect of each compound when used individually and to compare the results with the effect of whole BRBs extract both in vitro and in animal models.

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