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Assessment of Rutin's Anti-Metastatic Potential: Targeting the CXCL8/CXCR2 Chemokine Signaling Pathway in Oral Cancer Cell Line

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Abstract

Rutin is a flavonoid compound that is naturally found in many plant-based foods such as citrus fruits, buckwheat, and apples. It has been studied for its potential anti-cancer effects. The aim of the study is to investigate the anti-cancer effect of Rutin by targeting the CXCL-8/CXCR2 signaling pathway in oral cancer cells. To evaluate the cytotoxic potential of Rutin we have performed an MTT assay. A phase contrast microscope is used to evaluate changes in cell morphology. A scratch wound healing assay was performed to evaluate anti-migrative potential of rutin. Gene expression analysis was performed using quantitative real-time PCR to determine the levels of CXCL-8/CXCR2 signalling molecules. In our study, the exposure of oral cancer cells to rutin led to a notable decrease in cell viability, with a statistically significant difference observed ($p < 0.05$) between the control and treatment groups. The inhibitory concentration (IC-50) was determined to be 80 µg/ml for oral cancer cells. Post-treatment, a reduced number of cells were observed, exhibiting shrinkage and cytoplasmic membrane blebbing. The investigation indicated a significant downregulation of CXCL-8/CXCR2 mRNA expression following rutin treatment. Additionally, the migration of cells was significantly reduced compared to control cells. The results demonstrated that the Rutin was cytotoxic and inhibited cell migration by targeting the CXCL-8/CXCR2 signaling molecules gene expression in oral cancer cells. However, more research is needed to understand the mechanisms of the anti-cancer potential of this Rutin, by targeting the CXCL-8/CXCR2 signaling pathway might help to treat cancer.

Keywords: *CXCL-8/CXCR2, Chemokines, Metastasis, Oral cancer, Rutin.*

Introduction

Cancer remains a formidable health challenge worldwide, necessitating continuous exploration of novel therapeutic avenues. Until the 1970s, chemotherapy served as the second line of therapy subsequent to surgical amputation for cancer treatment [1]. The National Cancer Institute (NCI) outlines surgery, chemotherapy, radiotherapy, the use of samarium, and targeted cancer therapies as conventional approaches for cancer treatment, each carrying its drawbacks and side effects. Historically, effective chemotherapeutic drugs like doxorubicin, causing DNA damage in cancer cells, and cisplatin, inducing cell death through DNA adduct formation, have been employed. A diverse range of medications and pharmacological formulations have been explored in research for therapeutic interventions [2]. However, chemotherapy

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poses challenges such as side effects, increased recurrence rates, and the emergence of drug resistance. Consequently, researchers are actively seeking alternative treatments. Given the historical use of natural compounds in traditional Indian and Chinese medicine, global academics are focusing their research on substances and extracts derived from natural sources [3].

Rutin, a natural flavonoid, has garnered attention for its potential anticancer properties. Rutin is a flavonoid compound that is naturally found in many plant-based foods such as citrus fruits, buckwheat, and apples [4, 5]. Rutin, known for its antioxidant and anti-inflammatory attributes, emerges as a promising candidate for cancer intervention[6]. It has been studied for its potential anti-cancer effects in human carcinoma cells [4]. Oral cancers are formidable challenges in the realm of oncology, necessitating a deeper understanding of their molecular underpinnings for the development of effective therapeutic strategies.

Chemokines, small signaling proteins with pivotal roles in cancer progression and metastasis, [7] include the C-X-C motif chemokine ligand 8 (CXCL-8) and its receptor, C-X-C motif chemokine receptor 2 (CXCR2), implicated in oral cancer metastasis. Cancer cells secrete CXCL-8, acting as a chemoattractant for CXCR2-expressing cells, thereby fostering tumor growth, angiogenesis, and metastasis [8]. A notable multifunctional cytokine regulating tumor invasion and proliferation is CXCL8. Research highlights the involvement of CXCL8 and its receptors, CXCR1 and CXCR2, in various cancers like colorectal carcinoma, breast cancer, prostate cancer, lung cancer, and melanoma. CXCL8, interacting with diverse intracellular signaling pathways, plays a crucial role in the tumor microenvironment [9] by promoting neovascularization, a foundation for tumor development and metastasis. The interaction between CXCL-8 and CXCR2 activates downstream signaling pathways, enhancing cancer cell migration, invasion, and survival. Therefore, the present study aimed to evaluate the antimetastatic potential of rutin via targeting the CXCL-8/CXCR-2 Chemokine signaling in oral cancer cell line.

Understanding the CXCL-8/CXCR-2 axis in oral cancer holds promise for identifying novel therapeutic targets and refining treatment modalities. Insights gained from this exploration may pave the way for the development of targeted interventions that could potentially revolutionize the management of these challenging malignancies. In this study, we delve into the intricate landscape of Rutin's effects on cancer cells, with a particular focus on its modulation of the CXCL-8/CXCR-2 signaling pathway in oral cells. Understanding the molecular mechanisms underlying Rutin's anticancer effects can provide valuable insights for developing targeted therapeutic strategies.

Material and Methods

Cell Line Maintenance

From the NCCS in Pune, oral cancer cell lines (KB-1) were obtained. The cells were cultured in T25 culture flasks that included 10% FBS and 1% antibiotics in addition to DMEM. Cells were kept at 37 degrees in a humid environment with 5% CO2. The cells were trypsinized and passaged after they had reached confluence.

Cell Viability (MTT) Assay

Cancer cells that had been treated with Rutin had their cell viability measured using the MTT technique. In order for the test to work, metabolically active cells must convert soluble yellow tetrazolium salt into insoluble purple formazan crystals. KB-1 cells were seeded in 96-well plates at a density of $5x10^3$ cells per well. 24 hours after plating, cells were starved by incubating them for 3 hours at 37°C in serum-free media. Cells were then washed twice with 100µl of serum-free medium. Cells

were starved before being exposed to various Rutin concentrations for 24 hours. DMEM (0.5 mg/ml)-containing MTT was added to each well once the treatment was complete, along with 100 µl of media from the control and treated cells. The cells were next left incubated for 4 hours. After discarding the MTT-containing medium, after the created formazan crystals were mixed with dimethyl sulfoxide (100µl), a micro ELISA plate reader was used to measure the colour intensity at 570 nm. As a percentage of control cells grown in serum-free media, cell viability was calculated. Cell viability in the control, untreated medium was estimated using the following formula and represented as 100%: The formula for percentage cell viability is [A570 nm of treated cells / A570 nm of control cells] x 100.

Morphology Study

We chose the best doses (IC-50: 80µg/ml) for further research based on the MTT assay. A phase contrast microscope is used to examine changes in cell morphology. In 6 well plates, 2 10^5 cells were plated and given a treatment for cancer cells at a concentration of 80 µg/ml for 24 hours. The medium that was used was taken out of the cells after the time of incubation and they were given a single rinse in phosphate buffer saline (PBS, pH 7.4). With the use of a phase contrast microscope, the plates were examined.

Cell Migration Analyzed by Scratch Wound Healing Assay

Human oral cancer cell line (2×10^5 cells/well) was seeded onto six-well culture plates. The cell monolayer was scratched using a 200µl tip to create a wound. The detached cells were removed by washing with 1X PBS and add fresh culture medium with rutin for 24 h along with control group for 24 h. After incubation, the wells were washed and fixed in 4% paraformaldehyde. Photographs were taken using an inverted microscope (Euromex, The Netherlands).

TGFβ / Smad2 mRNA Expression by Real Time PCR

Real-time PCR (RT-PCR) was used to evaluate the gene expression of the TGFβ / Smad2 genes. Total RNA is isolated using a standardized protocol (Sigma) and trizol (Sigma) reagent is used. 2 µg of RNA is used to reverse-transcribe cDNA (cDNA synthesis) using PrimeScript (First Strand CDNA Synthesis Kit, TakaRa Japan). Target genes are amplified using specific PCR primers. The PCR reaction is performed using the PCR Master Mix (GoTaq®, qPCR) (Promega). This Master Mix contains a green SYBR dye and all of the PCR components. RT-PCR (Real-time PCR) is used to evaluate the results. CFX96 PCR is used in real-time. Biorad is used for real-time analysis. The fold change graph was calculated using the following methods: comparative CT method and $2-\Delta\Delta CT$ method.

Statistical Analysis

All data were acquired, reported as mean SD for triplicates, and analysed using one-way ANOVA and Student's t-test in SPSS. A $p< 0.05$ cutoff was used to determine statistical significance.

Results

Effect of Rutin on Cell Viability and Cell Morphological Changes of Oral Cancer Cell Line

The cell viability test was used to determine the cytotoxic potential of rutin. Various rutin concentrations were applied to the cells for 24 hours. Oral cancer cell viability was dramatically reduced by the rutin at the 24-hour mark as compared to controls. With an increase in rutin concentration, the proportion of viable cells steadily decreased. It was shown that a dosage of 80µg/ml for oral cancer cells resulted in a 50% suppression of cell growth (Figure 1). Therefore, the dosage described above was employed in subsequent studies. In comparison to the untreated cells, the KB-1 cell line underwent a 24-hour treatment with 80 µg/ml

of rutin and the results showed significant morphological changes (Figure 2). Cell atrophy and diminished cell count, both indicators of apoptotic cells, were among these modifications. In addition, cells experiencing death displayed additional changes in shape, such as rounder cells that shrunk and lacked a connection to neighbouring cells. A few delicate cells were also separated from the plates' surface.

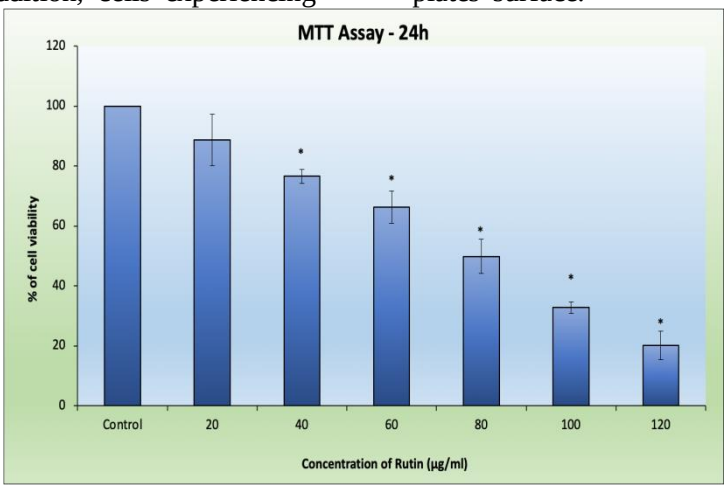

Figure.1. The Cytotoxic Effects of Rutin on Oral Cancer Cells

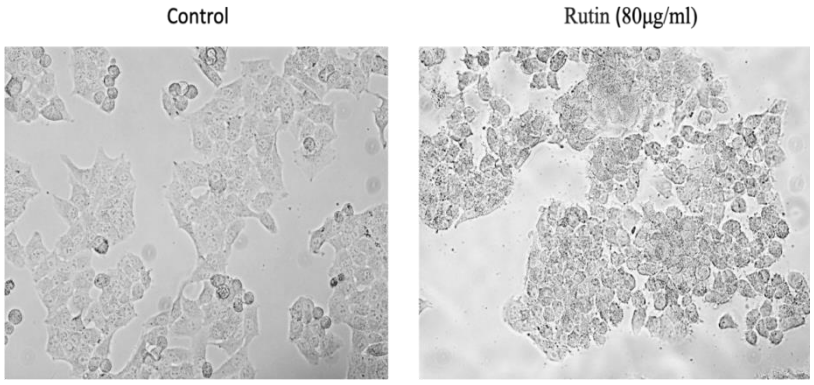

Figure 2. Effect of Rutin on Cell Morphology of Human Oral Cancer Cell Line (KB-1)

Inhibition of Cell Migration by Altering the TGF-B/SMAD2 Signaling Molecules on Oral Cancer Cell Line

Cancer metastasis is characterized by the migration of cells from one site to another, playing a crucial role in tumor invasion and spread. This migratory capacity is acquired by cancer cells through alterations in cytoskeletal dynamics and adhesion properties, enabling them to detach from the primary tumor and invade adjacent tissues. Investigating metastasis and cell migration in a controlled cell culture environment is instrumental in identifying potential therapeutic targets and devising innovative anti-cancer approaches. A scratch test was performed to evaluate the effect of rutin on the migration of oral cancer cells. The results showed that rutin inhibits the cell migration rate when compared to control cells. The following observations were made: In the control group, untreated cancer cells migrated to almost half of the scratched area after 24 hours. Treatment with rutin at a concentration of 80µg/ml significantly inhibited the migration of oral cancer cells compared to the control group. The migration distance of cells in the rutin group decreased compared with that observed in the control group (Figure 3).

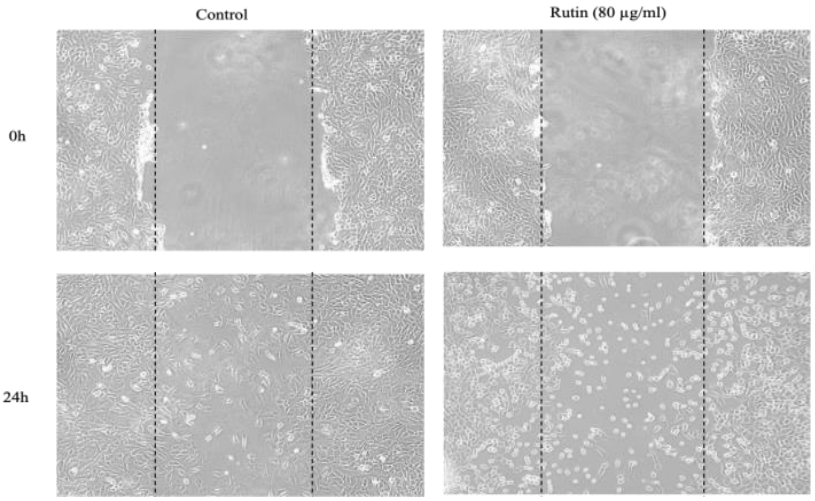

Figure 3. *In vitro* Scratch wound Healing Assay with and without Treatment of Rutin (80ug/ml)

The impact of rutin (80ug/ml) on the expression of the CXCL-8/CXCR2 gene in the oral cancer cell line was analyzed using Real-Time PCR, which offers important insights into the molecular mechanisms that underlie rutin possible anti-cancer capabilities. When the data are compared to the control group, they show a significant downregulation of CXCL-8 and CXCR2 gene expression, adjusted to GAPDH mRNA expression. Rutin at the prescribed dosage appears to have a strong inhibitory effect on the expression of these chemokines in oral cancer cells, as indicated by the fold change from the control, which shows a noticeable reduction of these chemokines. The reliability and significance of the observed changes are shown by the statistical significance (* at $p<0.05$). We observed that rutin treatment significantly inhibited TGF-β and SMAD-2 gene expression in cancer cells. This downregulation of CXCL-8/CXCR was associated with the inhibition of oral cancer cell migration (Figure 4).

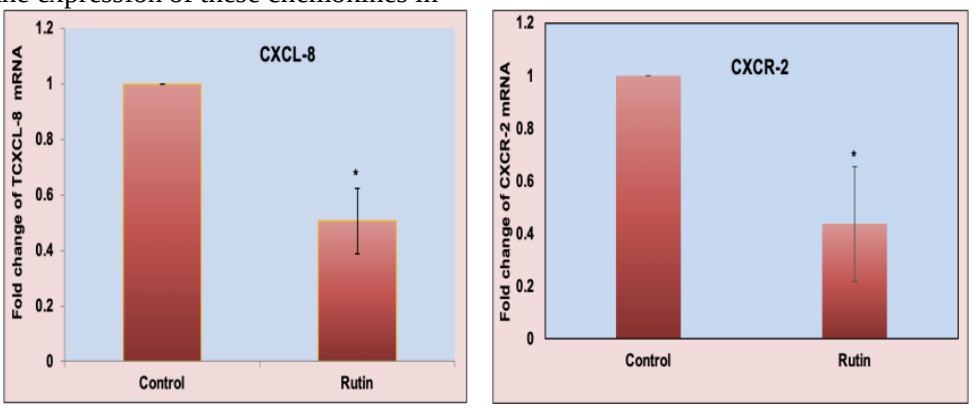

Figure 4. Effect of Rutin (80µg/ml) on CXCL-8/CXCR-2 Gene Expression in Oral Cancer Cell Line

Discussion

Oral cancer refers to cancers that develop in any part of the mouth, including the lips, gums, tongue, cheeks, and the roof or floor of the mouth. It is a type of head and neck cancer and can occur in various forms, including squamous cell carcinoma, which is the most common type. Risk factors for oral cancer include tobacco use (smoking or chewing), excessive alcohol consumption, human papillomavirus (HPV) infection, poor oral hygiene, and a diet low in fruits and vegetables. Treatment may involve surgery, radiation therapy, and/or chemotherapy, depending on the stage and type of cancer [10, 11].

The investigation into Rutin's anticancer properties and its impact on the *CXCL-8/CXCR-2* signaling pathway in oral cells has yielded noteworthy findings, shedding light on the potential therapeutic relevance of this natural compound. Our study reveals a compelling modulation of the *CXCL-8/CXCR-2* signaling pathway by Rutin. This pathway, known for its intricate involvement in cellular processes, appears to be a key target for Rutin's anticancer effects. The previous studies discuss various pharmacological activities of rutin, including its antioxidant and anti-inflammatory effects. However, the study does not provide conclusive evidence on rutin's specific impact on oral cancer [4, 12]. Another study investigates the chemopreventive effect of rutin in a mouse model of skin carcinogenesis. While the results suggest a potential preventive effect, the relevance to oral cancer or other types of cancer needs further exploration [13].

Rutin, a natural flavonoid compound, has been under scrutiny for its ability to disrupt this crucial pathway. The findings from the assessment reveal promising anti-metastatic effects of rutin, laying the foundation for further discussion. Primarily, the study underscores the impact of chrysin on cell viability. The cells were treated with different concentrations of rutin and it was found that with increase in concentration there is gradual decrease in cell viability, This suggests a potential cytotoxic effect on oral cancer cells, aligning with the hypothesis that rutin may impede the metastatic capabilities of these cells. Thus, it is essential to create new medications that are risk-free, efficient, and have few adverse effects [14–16]. In this study, we looked into rutin cytotoxic and anti-migratory effects on the oral cancer cell line. Initially, the oral cancer cell line was subjected to varying concentrations of rutin (20 – 120 µg/ml) for 24 hours to evaluate its inhibitory effect on the growth of oral cancer cells. Our findings demonstrated that rutin administration dramatically reduced KB-1 cells' viability in a dose-dependent manner. The IC50 value of 80 µg/ml for oral cancer cells was selected to further assess the inhibitory impact, and the morphology was examined using a phase-contrast microscope to confirm its anticancer potential.

Microscopic examination further corroborates these observations, unveiling signs of cell shrinkage, membrane blebbing, and a reduction in cell count following rutin treatment when compared to untreated cells. Here, the oral cancer cells were significantly reduced after treatment with rutin for 24 hrs and the cells exhibited the indications of cytotoxicity by shrinking and blebbing of the cytoplasmic membrane. The most well-established anticancer strategy involves inducing apoptosis in tumour cells, and it is used in numerous cancer treatments [14, 17]. Rutin may have an anti-metastatic impact because of the downregulation of CXCL-8, a pro-inflammatory chemokine linked to tumor growth and metastasis, and its receptor CXCR2. The chemokine expression regulation is consistent with the anti-inflammatory and anti-cancer capabilities of rutin that have been previously documented. The findings imply that rutin may disrupt signaling pathways implicated in the advancement of cancer, obstructing the recruitment of inflammatory cells and reducing the capacity of oral cancer cells to metastasize [18, 19]. Rutin potential as a therapeutic agent in the treatment of oral cancer is highlighted by the downregulation of CXCL-8 and CXCR2 gene expression, which offers a mechanistic basis for the reported anti-cancer actions [20]. The observed alterations in CXCL-8 and downstream events underscore Rutin's potential to influence critical signaling cascades implicated in cancer progression. The significant antiproliferative effects observed in oral cancer cells following Rutin treatment corroborate the potential therapeutic utility of this flavonoid. The suppression of cell proliferation aligns with the notion that Rutin

may impede the uncontrolled growth characteristic of cancer cells [4].

Conclusion

In conclusion, our study advances our understanding of Rutin's anticancer properties, particularly its modulation of the *CXCL-8/CXCR-2* signaling pathway. The multifaceted effects observed in oral cells emphasize the potential of Rutin as a targeted therapeutic agent in the realm of cancer treatment. As the research community continues to unravel the intricacies of Rutin's molecular interactions, there is optimism for its translation into innovative and effective cancer therapies.

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Conflict of Interests

Authors have declared that no competing interests exist.

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