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Quercetin Contained in TCM Causes CNS Cancer Cell Apoptosis by Activating the Mitochondrial Pathway of Apoptosis

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Abstract

Objective: The previous research done by Shikha Srivastava et al. proved that the natural flavonoid, quercetin is able to cause apoptosis by activating the mitochondrial pathway in Nalm6 cells for breast cancer. However, the flavonoid's effectiveness on CNS cancer and the exact triggers are not fully explored. This study seeks to discover the difference of effects of quercetin on CNS cancer cell lines (T98G, U-87, U-251) and the reasons why these effects exist. Methods: This research utilizes the flow cytometry analysis to perform apoptosis analyses on the CNS cancer cell lines. Additionally, annexin V and PI binding on the cell lines is also tested, with results retrieved from a flow cytometry analysis. The MTT assay and western blot will also be utilized to analyze the rate of apoptosis and the cleavage of CASPASE 9, 8, and 3. Possible Results: There are 11 possible results proposed in this research paper, with 1 result completely agreeing with the hypothesis. Conclusion: The results of this study will further investigate the effects of quercetin in CNS cancer cell lines and allow a deeper understanding of the responsive apoptosis process for CNS cancer cells. Further research should explore the clinical usages of quercetin and/or other more effective pathways that trigger different forms of cell death in cancer cells.

Keywords

Quercetin; TCM; CNS Cancer; Apoptosis; Mitochondrial Pathway.

1. Introduction

There is no doubt, cancer is certainly one of the world's most deadly and feared diseases. However, unlike what legends make it sound, they actually have many potential cures [1]. Nonetheless, it is still hard to treat and comes in many distinct forms. One of the most dangerous form of cancer is the CNS cancer or central nervous system lymphoma. It is a disease where cancer cells form inside the lymph tissues on the brain or spinal cords, severely threatening our body's control center [1]. Although there are methods of remission, the solutions are not perfect. Current treatments to this disease include the whole brain radiation therapy, targeted chemotherapy, or clinical trials that consist of both and includes additional stem cell transplants [1]. Therefore, the question becomes obvious. Being a disease that is so deadly and frightening, are there really no treatments that can naturally cause remission? Luckily, there is.

Not known to a lot of people, a molecule inside traditional Chinese medicine used in common hospitals may be used to cause cancer cell remission. Quercetin is a natural flavonoid that is very commonly found in traditional Chinese medicine, vegetables, as well as fruits. Surprisingly, it can actually be used effectively as a cytotoxin to cancer cells that reduces the multiplication of these cells, resulting in cancer repression [2]. In the previous study done by Shikha Srivastava et al., quercetin has been proven to be able to induce apoptosis through enabling the mitochondrial apoptosis pathway in two different cancerous cell lines, which ends the constant reproduction of cancer cells and triggers cell death [3]. (Srivastava, 2016) Moreover, this previous study also shows that quercetin's special

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property can also reduce the size of cancerous tumors in mice subjects. However, this research is specifically done on leukemia and breast cancer cells.

Overall, the prediction is that quercetin can be used to arrest cell cycle and cause apoptosis in CNS cell lines (T98G, U-87, U-251) by enabling the mitochondrial apoptosis pathway, resulting in tumor size regression and apoptosis [3]. The current research proposal will seek to explore the usages of quercetin in CNS cancer (central nervous system lymphoma) and try to analyze whether it can have the same effect of inducing apoptosis on the cancerous cells. Similar to the cells Nalm6 used in the previous study [3], the CNS cell lines used in this study (T98G, U-87, U-251) are also cancerous. However, as proven by the national cancer institute using the SRB assay, these cell lines have a significantly higher sensitivity to quercetin treatments, which will most likely increase the probability of cancer suppression [2]. Nonetheless, this is a raw piece of information, and there are no direct explanations for any of the reasoning behind it. Although the SRB assay proved that CNS cell lines are generally more sensitive, it does not describe any of the processes of its apoptosis and its causes. Therefore, this research paper will seek to analyze the exact mechanisms of why CNS cancer cells can be suppressed by quercetin, which results in apoptosis. Utilizing existing information about cervix cancer cell lines and quercetin [3], this study will develop a conclusion examnining exactly how and why quercetin is able to remiss CNS cancer in cell lines.

2. Hypothesis

I predict that quercetin can be used to arrest cell cycle and cause apoptosis in CNS cell lines (T98G, U-87, U-251) by activating the mitochondrial apoptosis pathway. The apoptosis assays of CNS cell lines will be measured through incubating the cell at 37 °C with quercetin for an increasing amount of time and quercetin concentration. The results will come from the flow cytometry analysis [3]. (8, 16, 24h) (1μM, 10μM, 100 μM, 250 μM). PI/Annexin V staining and MTT assays will be applied. Caspase 9/3/8 cleavage will also be tested by western blot. If capsase 9 cleaved, then it is triggered from the mitochondria. If caspase 8 is cleaved while caspase 9 is not cleaved, then the apoptosis process would be triggered from death receptors. The tumor size regression will be measured using EAC cell lines in xenograft models. Taxol will be used as a positive control and will be used on the cancer cells, the negative control will be no quercetin.

3. Methods/Materials

This experiment will be repeated for three times. The significance will be measured using the student T test for a P value of less than 0.05.

Cell Lines: Human CNS cancer cell lines (T98G, U-87, U-251) will be purchased from the ATCC cell biology collection.

Flow Cytometry Analysis: In order to conduct the apoptosis assay of the CNS cells, a cell cycle analysis will be done in U-87 cell line. The cell line will be treated with quercetin (10 μM) for (8, 16, 24h). This will examine the effects of quercetin for an extended period of time. Another set of experiment will test the effects of an increased amount of quercetin. The cell line will be treated with an increasing amount of quercetin (1μM, 10μM, 100 μM, 250 μM) for 24 h. In all experiments, cells will be seeded at 0.75 × 105 cells/ml [3,2]. Cells will then be stained with propidium iodide to be analyzed by the flow cytometry analysis.

PI/Annexin V binding: To recognize apoptosis, the cell lines will be incubated with quercetin, harvested, stained with Annexin V which will bind with phosphatidylserine that exist in apoptosis cells to detect apoptosis and PI (propidium iodide) which is used to identify stained cells. The results will be analyzed using a flow cytometry analysis which allows the fluorescent of Annexin V to be seen [4]. This will allow the researcher to understand the amounts of cells that experienced apoptosis after being incubated with quercetin.

MTT Assay: MTT Assay will be used to analyze the metabolism of the CNS cell lines in order to measure the effects of quercetin in cell death. The three cell lines will be seeded at 0.75 × 105 cells/ml

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for 24 h, and then treated with increasing amounts of quercetin (1μM, 10μM, 100 μM, 250 μM) for 48 h [3,2]. Afterwards, the cell lines will be subjected to MTT Assay.

Western Blot: The CNS cells will be seeded at 0.75 × 105 cells/ml, harvested after their quercetin treatments (1μM, 10μM, 100 μM, 250 μM), and each prepared in RIPA buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) [1]. Then, the membrane will be incubated with antibodies (CASPASE 9, CASPASE 3, CASPASE 8) at 4 °C overnight [3]. Then, the western blot will be performed to analyze whether the CASPASE 9 is cleaved. If CASPASE 9 is cleaved after the quercetin treatment, the apoptosis is triggered from the mitochondrial pathway. However, if CASPASE 9 is not cleaved while CASPASE 8 is cleaved, then the apoptosis is triggered from death receptors instead of the mitochondria [5].

4. Hypothesized Results

Table 1. Possible results of the experiment comparing quercetin treated to untreated cells

Quercetin Treated CNS Cell Lines	Experimental Procedures		
	Annexin V	PI	MTT (Cell Death)
Result 1	++	++	++
Result 2	--	++	++
Result 3	++	--	++
Result 4	--	--	++
Result 5	++	++	--
Result 6	--	--	--

Result 1: Quercetin exhibits cytotoxicity in CNS cancer cell lines, which are results of apoptosis inside of the cells.

The three CNS cancer cell lines (T98G, U-87, U-251) will show significant decrease in metabolic activities, as examined by the MTT assay. Therefore, quercetin does have a cytotoxicity effect on the cell lines. Moreover, the Annexin V/PI staining also shows that the cell death is caused by apoptosis.

Result 2: Quercetin does exhibit a degree of cytotoxicity in CNS cancer cell lines but may be a result of necrosis instead of apoptosis.

As shown by the MTT assay, quercetin does exhibit a degree of cytotoxicity in the CNS cell lines and causes cell death. However, since the result of Annexin V staining is negative, although the PI staining results positive, it can be inferred that the cells are not experiencing apoptosis, but instead, necrosis cell death.

Result 3: Quercetin does exhibit cytotoxicity in CNS cancer cell lines and cause early apoptosis in cells.

The MTT assay showed that quercetin does exhibit cytotoxicity in CNS cancer cell lines and causes cell death. However, because the Annexin V bonding is successful while the PI bonding results in negative, the cells is probably experiencing early apoptosis.

Result 4: Quercetin does exhibit cytotoxicity in CNS cancer cell lines but does not cause the two form of cell death explored in this research (apoptosis or necrosis).

The MTT assay displays that quercetin does display a level of cytotoxicity in CNS cancer cells as some cell deaths appear. However, since neither Annexin V or PI bonding were positive, it will not experience apoptosis or necrosis.

Result 5: Quercetin does not exhibit cytotoxicity in CNS cell lines but can cause late apoptosis in cells.

After the MTT assay, quercetin is showed to not result in clear cell death for the CNS cell lines. Therefore, the conclusion for it having significant cytotoxicity cannot be proven positive. However,

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as both the Annexin V and PI staining have resulted in positive, it can be inferred that it does cause late apoptosis in the cell lines.

Result 6: Quercetin does not exhibit cytotoxicity in CNS cell lines and does not cause apoptosis in the cells.

The MTT assay showed that quercetin does not cause cell death for the CNS cancer cells. This means that quercetin does not have significant cytotoxicity for these cells. Moreover, since the Annexin V and PI staining also resulted negatively, quercetin also cannot be proven to cause apoptosis or necrosis in cells. However, this may also be attributed to the assays malfunctioning if the Taxol controlled positive test also displayed a negative result.

Table 2. Results of CASPASE 9/8/3 Cleavage in CNS cancer cell lines

Quercetin Treated CNS Cell Lines	CASPASE Cleavage (With ++ Annexin V Results)		
	CASPASE 9 (Cleavage)	CASPASE 3 (Cleavage)	CASPASE 8 (Cleaved)
Result 7	++	++	--
Result 8	++	++	++
Result 9	--	++	--
Result 10	--	--	++

Result 7: Quercetin cause apoptosis in CNS cancer cell lines through activating the mitochondrial apoptosis pathway.

Additionally to the Annexin V and PI results proving quercetin's ability to cause apoptosis, CASPASE 9/3 being cleaved while CASPASE 8 remaining not cleaved means that the apoptosis of CNS cancer cells is triggered from its mitochondria.

Result 8: Quercetin cause apoptosis in CNS cancer cell lines through activating the mitochondrial apoptosis pathway.

The western blot proves that although CASPASE 9 and 3 are both cleaved by the quercetin treatment, CASPASE 8 is also cleaved during the process. This means that the apoptosis is resulted from the mitochondria.

Result 9: Quercetin does not cause apoptosis in CNS cancer cell lines through the mitochondrial apoptosis pathway

The western blot results display that although CASPASE 3 is cleaved during the quercetin treatment, CASPASE 9 is not cleaved. Additionally, CASPASE 8 is also not cleaved, meaning the apoptosis is not triggered from the mitochondria.

Result 10: Quercetin cause apoptosis in CNS cancer cells, but it is not triggered from the mitochondria, but from death receptors

Although this result still displays quercetin's ability to cause apoptosis, the negative CASPASE9 cleavage results mean that the apoptosis is not triggered by the mitochondria. Additionally, CASPASE 8 being cleaved means that the apoptosis is not enabled from the mitochondria, but from death receptors.

Result 11: Quercetin is able/unable to cause apoptosis in CNS cancer cell lines, but cannot reduce tumor size

This result is an addition to all the previous results. If quercetin cannot reduce the size of cancerous tumors, the result of the xenograft models will come back negative.

5. Discussion

Unlike other forms of cancer, the CNS cancer directly damages the central nervous system, resulting in the spread of cancerous cells to the brain, eye, or even the whole spinal cord. Therefore, it is definitely important to know that quercetin treatment will have a positive effect on this specific type

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of cancer. Previous studies done by Shikha Srivastava et al. proves that quercetin treatment is effective in the remission of breast cancer [3]. Similarly, this study asks whether quercetin treatment is also effective in the remission process of CNS cancer by directly activating cell apoptosis for the CNS cancer cells.

The main result (result 1) of this study will show that as quercetin enters the cell, it will result in caspase 9 cleavage, which will further result in cleavage in caspase 3 and other proteins, finally causing a reaction in the mitochondrial apoptosis pathway. This will lead to the mitochondrial pathway causing apoptosis for CNS cancer cells. This result exhibits quercetin's ability to ease CNS cancer development and cause regressions in cancerous tumors. Through this study, the hypothesis proposed by Shikha Srivastava et al. is further expanded. Instead of being able to remiss breast cancer cell development, quercetin is also proven to be able to cause apoptosis in CNS cancer cell lines as well. Moreover, the cytotoxicity of cells will be proven through MTT assays. Among the different CNS cell lines, T98G will exhibit the most significant cell death. Therefore, it will be the most sensitive of all the cell lines chosen. Moreover, it will be proven that the CNS cancer cell lines will experience cell apoptosis in a dose-dependent manner, as the cell death will become more significant as the doses of quercetin increases.

To analyze whether the previously proven cytotoxicity will lead to apoptosis, the Annexin V/PI staining technique is used. Annexin V will bind to phosphatidylserine, which exists commonly in cells experiencing apoptosis [4]. Normally, the phosphatidylserine will only exist on the cytosolic side of the cell membrane. However, during apoptosis, the cell shifts and moves the phosphatidylserine to the outer layer of the cell membrane, making it easy for Annexin V enzyme to bind to it [4]. Therefore, each of the CNS cell line sample treated with increasing amounts of quercetin (1μM, 10μM, 100 μM, 250 μM) for increasing amounts of time (8, 16, 24h) will be stained with Annexin V and examined using the flow cytometry analysis.

The main difference between result 1 and 2 is that since Annexin V staining resulted negatively while PI staining remained positive, quercetin may not result in apoptosis, but will result in necrosis. The ineffective binding can be resulted in a few different reasons. First, human errors like sample infection, wrong timing, or sample spillage can all contribute to an inaccurate result. Second, the annexin-binding buffer may not be applied or is applied but showed minimal affect. This will also influence the final result as without proper buffer, the difficulty of annexin V binding to phosphatidylserine will decrease, resulting in a lower rate of discovering apoptotic CNS cells. Additionally, there is also the potential of quercetin not being able to induce phosphatidylserine exposure on CNS cell surfaces, which will definitely not cause apoptosis.

Similarly to result 1, result 3 does allow the CNS cell lines to experience apoptosis (due to Annexin V bonding) and causes cytotoxicity in cells. However, one main difference is that the PI bonding resulted negatively. This can be attributed to insufficient bonding buffer, human errors causing cell infection, or the quercetin simply not being able to form bonds with PI molecules. Without proper PI bonding, the result will display cells that are experiencing early apoptosis instead of late for double positive results.

Result 4 is a case where the cell death is seen without clear signs of apoptosis or necrosis occurring in the cells. Since neither Annexin V or PI bonding is proven positive by the flow cytometry analysis, it is clear that the CNS cell lines are experiencing neither apoptosis (Annexin V positive) or necrosis (PI positive).

Result 5 is similar to result 1 as they all showed quercetin's ability to cause apoptosis (late apoptosis). However, since the MTT assay resulted negatively in result 5, the cytotoxicity of quercetin to CNS cell lines cannot be proven as there are no clear cell deaths. Nonetheless, since the annexin V and PI bonding all resulted in positives, it can be inferred that quercetin molecules do result in apoptotic cells, since the annexin V formed phosphatidylserine bonds successfully with the cell surfaces, the cells are most likely experiencing apoptosis.

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Result 6 is a case of all negatives. Since the MTT assay displayed quercetin not having any ability in causing cell death, it does not appear to be cytotoxic to CNS cancer cells. Moreover, since the Annexin V/PI also resulted in negatives, the quercetin molecule most likely did not cause apoptosis, since the phosphatidylserine bonds between the cell surface and Annexin V was not formed successfully. However, these results may all be attributed to human errors if the result of the positive control also comes in as a negative.

Result 7-10 only involve the enzymes CASPASE 9, 8, and 3. CASPASE 9 and 3 cleavage indicate apoptosis caused by CASPASE 9 cleavage, which turn cleaves CASPASE 3 and leads to degradation of cell DNA, leading to apoptosis [3, 5]. On the other hand, CASPASE 8 is a caspase protein that indicates whether the relation between the apoptosis and the mitochondria exists [5]. If CASPASE 8 is cleaved through the quercetin process while CASPASE 9 and 3 are not cleaved, then the apoptosis is not triggered from the mitochondria, but instead from death receptors [5].

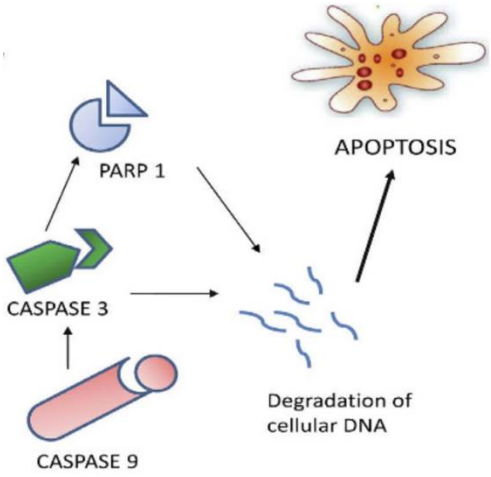

Figure 1. Explanation diagram from Shikha Srivastava et al. [3]

Result 7 is quite straight forward, with CASPASE 9 and 3 confirming the apoptosis in CNS cell lines from the quercetin and un-cleaved CASPASE 8 confirming the hypothesis that the apoptosis is indeed resulted from the mitochondria. Result 8 is similar, since positive CASPASE 9 result means mitochondrial apoptosis no matter that the CASPASE 8 result is positive or negative, this result also shows that the apoptosis is triggered from the mitochondrial. Result 9 shows an alternative that disproves the mitochondrial apoptosis through the un-cleaved CASPASE 9. However, since the CASPASE 8 is also un-cleaved, the apoptosis is most likely not resulted from death receptors either. The 10th and final result shows a possibility that the apoptosis is triggered from the death receptors instead of the mitochondria since the CASPASE 8 is cleaved while CASPASE 9 and 3 are both un-cleaved.

Result 11 is an addition to the previous results. After the xenograft models come out, if the tumor size regression is not present, then it can be deduced that quercetin may not have the ability to reduce tumor size. This result may be attributed to quercetin not having the ability to reduce tumor size or human/xenograft model error.

However, the results of this study are limited, as it only allows the researcher to understand that the apoptosis is caused by the mitochondrial pathway (caspase 9 cleavage) and does not go into further detail describing how exactly does the proteins react to the cleavage and how it eventually lead to the apoptosis. Therefore, although the original hypothesis is proven, the explanation is not complete, and there are still ideas left to be proven.

Further research should seek to analyze similar flavonoids and their ability to cause apoptosis in different cancerous cells. Since quercetin is able to cause apoptosis, flavonoids that have similar molecular structures should be able to function in a similar way. Moreover, further researchers can

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also explore how to strengthen the effects of the quercetin treatment and allow it to become a more relevant clinical application for cancer treatments.

Table 3. Results Analysis

Result #	Effectiveness of Supporting Hypothesis
Result 1	Partially support hypothesis
Result 2	Partially support hypothesis
Result 3	Partially support hypothesis
Result 4	Partially support hypothesis
Result 5	Partially support hypothesis
Result 6	Contradict hypothesis
(Results 7-9 all fully support the first section of the hypothesis: "quercetin can be used to arrest cell cycle and cause apoptosis in CNS cell lines")	
Result 7	Fully support hypothesis
Result 8	Fully support hypothesis
Result 9	Partially support hypothesis
Result 10	Partially hypothesis
Result 11	Contradict hypothesis

6. Conclusion

Compared to other fields, the treatment of cancer is still a study full of possibilities. Because of the limited treatment methods of the current medical field, a natural remission for cancer will be extremely innovative and beneficial not only for cancer patients, but for the entire field of medicine. This research question is important as it explores the effectiveness of a natural flavonoid on a specific cancer and explains exactly why it will result in the cell deaths of cancer cells. This will allow clinical experts to gain a new perspective on quercetin treatments and allow researchers to further explore the biochemical reasons behind quercetin's ability to remiss the CNS cancer.

Additionally, alternative hypotheses of this research also include quercetin's ability to cause anti-inflammatory effects or strengthen the immune system. Nonetheless, one of the most practical reasons behind quercetin's ability to remiss cancer is its ability to cause apoptosis in cells, which is the main focus of this research. In the future, the effects of quercetin on cancer cells will hopefully be further explored, including not only how it affects cancerous cells, but also the healthy cells in the body. If further researches are successful, quercetin may be able to become one of the most innovative and natural solutions for cancer remission and be applied clinically.

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