The anti proliferative effects of rosemary oil on keratinocyte cell line, HaCaT

Muhammet Kuddusi ERHAN*

Department of Crop and Animal Production, Vocational High School, Ağrı İbrahim Çeçen University, Ağrı, Turkey

Abstract: This study aims to investigate the action of rosemary oil on the immortalized keratinocyte cell line. Samples of *Rosmarinus officinalis* that were growing uncultivated in central Turkey were harvested during flowering in May 2016. The rosemary oil was obtained by the hydrodistillation of the dried ground material in a Clevenger-like apparatus. Human cells from the immortalized nontumorigenic keratinocyte cell line were seeded at a concentration of 5000 cells/well on 96 well plates. Cell viability was measured at the 24, 48, and 72 h. Five different doses of 1.25% to 20% *Rosmarinus officinalis* oil, 20% DMSO (for toxic examination), and 1 negative control were prepared. Highly significant differences were found between the doses ($P < 0.01$) as well as at different time points ($P < 0.01$). That is to say that at 72 h, the results for mean ± SD cell vitalities for doses of 1.25, 2.5, 5, 10, 20, DMSO 20%, and the control were 0.099 ± 0.018, 0.077 ± 0.008, 0.038 ± 0.011, 0.022 ± 0.005, 0.011 ± 0.000, 0.009 ± 0.000, and 0.174 ± 0.011, respectively. There was a significant decrease in the mean vitality from baseline (3.4%) to 48 h (2.0%) followed by a substantial increase at 72 h (6.1%) ($P < 0.01$). This study shows that rosemary oil used in cell culture inhibits cell proliferation, which might be via the apoptotic pathway. We concluded that rosemary oil at a concentration of 1.25% may help the organism to inhibit excessive cell proliferation in tissues covered with keratinocytes, which may contribute to a healthy digestive system by stopping excessive cell proliferation and may be utilized in both animal feed and human nutrition, via the apoptotic pathway.

Key words: Animal feed, apoptosis, cell culture, keratinocyte cells, rosemary plant

1. Introduction

More recently, rosemary is examined using modern scientific methodology, and as a result of these investigations, a lot of useful information about the plant and its derivatives has been accumulated [1,2,3].

Rosemary (*Rosmarinus officinalis*, L.) is one of the significant medicinal plants belonging to the Lamiaceae family and has been widely used for different purposes traditionally, including treatment and nutrition.

It was claimed that rosemary extract exhibits a variety of pharmacological activities via its main chemical compound, which includes carnosol, carnosic acid [4,5], ursolic acid, rosmarinic acid [6], and caffeic acid [7,8]. Most of the recent studies aimed at finding a possible mechanism of rosemary oil concerning its effects on tumor cells. That is because the potential molecular mechanism of its antiproliferative effect and the pharmacological interactions among rosemary components are still unknown [9,10,11]. Therefore, we hypothesized that rosemary oil can induce apoptosis in cells by slowing down or stopping cell division, leading to cell death.

Keratinocytes constitute 90% of the outermost layer of the skin. These cells have a high capacity for proliferation, which is tightly controlled under physiological conditions [12].

Although the findings on the antiproliferative effect of rosemary are well appreciated, the mechanisms of action are not fully resolved. For example, in the in vitro study on colon cancer cells, Pérez-Sánchez et al. commented that the treatment with rosemary would lead to a heavy increase of intracellular reactive oxygen species (ROS) and high-level ROS could result in necrosis and cell death [10]. On the other hand, cytotoxic effects of rosemary will block nuclear factor erythroid 2-related factor 2 (Nrf2) gene expression. Thus, a sequence of events will be detected in cell survival [9].

This study aims to investigate the antiproliferative action of rosemary oil on the immortal keratinocyte cell line and contribute to a better understanding of the possible mechanism of this action.

2. Materials and methods

2.1. Plant material

Samples of rosemary spontaneously growing in Erzurum (39.9055° N, 41.2658° E, at an altitude of 1800 m above the mean sea level) were harvested during flowering in

* Correspondence: mkuddusi@hotmail.com

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May 2016. The samples were identified by the Department of Aromatic and Medicinal Plants Research, National Institute of Agricultural and Food Technology (INIA), Erzurum, Turkey. A voucher specimen was deposited for internal control at the INIA.

Samples were dried and were kept in an oven at 35 °C for 30 h. They were kept away from light and moisture until the analysis.

2.2. Extraction process

The rosemary oil was obtained by hydrodistillation of the dried ground material in a Clevenger-like apparatus for 2 h at atmospheric pressure of about 100 g of the sample. The time taken was marked from the falling of the first drop of the distillation. During the process, 2 replications were performed. The rosemary oil yield was evaluated gravimetrically. Based on the dry weight, the ratio was 1.7%

2.3. Cell culture

The immortalized nontumorigenic keratinocyte cell line of human (HaCaT) was obtained from the Cell Culture and Biological Resources Unit at Yeditepe University, Turkey. These cells were seeded at a concentration of 5000 cells/well on 96 well plates (BIOFIL, TPC, Switzerland) and maintained in RPMI-1640 (Sigma Chemical Co., St. Louis, MO), containing HEPES (Sigma) buffer supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (Hyclone Lab., Logan, UT), 100 U/ml penicillin (Sigma), and 100 µg/mL streptomycin (Sigma), in plastic disposable tissue culture flasks at 37 °C in a 5% CO_2 / 95% air incubator.

2.4. Cell viability

The cell viability was measured at 24, 48, and 72 h using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H tetrazolium measurement methods of cell proliferation (MTS) assay (CellTiter96 Aqueous One Solution, Promega, UK).

Five different concentrations of 1.25%, 2.5%, 5%, 10%, and 20% *Rosmarinus officinalis* oil, 20% dimethyl sulfoxide (DMSO) as a toxic control, together with a nothing-added negative control were prepared in Dulbecco's Modified Eagle Medium (DMEM) including 10% Fetal bovine serum (FBS) and 1% penicillin-streptomycin-amphotericin (PSA).

On the evaluation day, the MTS solution was prepared according to the manufacturer's instructions, and cells were treated with this solution. The plates were incubated for 2 h in the dark at 37 °C. Cell viability depending on the toxicity of *Rosmarinus officinalis* oil was measured by an ELISA plate reader (Biotek, USA) at 490 nm absorbance.

2.5. Statistical analysis

Each experiment was carried out in triplicate. The results were presented as mean ± standard deviation (SD) values of at least 3 measurements. A 2-factor (Dose and Hours) full chance trial plan was applied for both groups, and the one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between doses and hours. Differences among the means were determined by the Duncan multiple comparison test. A linear correlation analysis was used to explore the relationships among the study. Correlation coefficients (R) and P values were evaluated to judge the fit of the correlation. Two-sided P < 0.05 and P < 0.01 values of relationships were considered significant and highly significant, respectively. All analyses were conducted using the statistical package SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

According to the results of the variance analysis (Table 1), highly significant differences were found among the doses at 24 h for rosemary oil (P < 0.01), and there was no difference between 20% and 20% of DMSO (positive control). Additionally, there was still no significant difference between 2.5% and 5%, but the negative control, 1.25%, and 10% groups were significantly different (P < 0.01) from one another.

The values at 48 h showed significant differences among the doses (P < 0.01). There was no difference between 20% and DMSO 20% (positive control), or between 5% and 10%. However, the negative control, 1.25% and 2.5% groups were significantly different (P < 0.01) from one another (Table 1).

When the 72-h-data for rosemary oil were examined, significant differences were found again among the doses (P < 0.01). There was no difference between 20%, DMSO 20% (positive control), and 10%. However, 1.5%, 2.5%, 5%, and the negative control group were significantly different (P < 0.01), as shown in Table 1.

Also, there were significant differences between the mean cell vitality measured at different time points. There was a considerable decrease in the vitality at 48 h, followed by a substantial increase at 72 h (Table 2).

4. Discussion

We have already studied the antioxidant effects of rosemary in the previous section [13]. In this study, we want to move the first topic a step further and examine whether or not rosemary oil has antiproliferative effects on the immortalized human keratinocyte nontumorigenic cell line (HaCaT).

Although rosemary was investigated before, this study contributes to the contemporary investigations in some aspects. First, the effectiveness of rosemary was tested in normal cells, not in pathological cells. Instead of plant components, rosemary oil was used in the experiment directly. Additionally, rosemary's antiproliferative effect

Table 1. Distributions of mean cell vitality between the different experimental groups.

Dose	24 h	48 h	72 h
Toxic control DMSO 20%	0.011 ± 0.001[a]	0.008 ± 0.000[a]	0.009 ± 0.000[a]
Negative control	0.073 ± 0.108[e]	0.047 ± 0.160[d]	0.174 ± 0.011[e]
1.25%	0.054 ± 0.008[d]	0.029 ± 0.005[c]	0.099 ± 0.018[d]
2.5%	0.035 ± 0.005[c]	0.020 ± 0.001[b]	0.077 ± 0.008[c]
5%	0.030 ± 0.002[c]	0.014 ± 0.004[ab]	0.038 ± 0.011[b]
10%	0.022 ± 0.003[b]	0.014 ± 0.008[ab]	0.022 ± 0.005[a]
20%	0.013 ± 0.001[a]	0.009 ± 0.000[a]	0.011 ± 0.000[a]
P	<0.01	<0.01	<0.01

Similar subscript letters designate the absence of significance. DMSO: Dimethyl sulfoxide.

Table 2. Inter-hour variance analysis.

Hour	$\underline{X} \pm SS$
24	0.034 ± 0.001[b]
48	0.020 ± 0.001[a]
72	0.061 ± 0.001[c]
P	<0.01

was evaluated in an immortalized human keratinocyte nontumorigenic cell line (HaCaT) in vitro. On the other hand, the lack of generalizability in human subjects can be mentioned as a limitation of this study.

Due to their high proliferative abilities, epithelial cells are capable of regenerating themselves continuously at almost every stage of postnatal life, just like in the development process. The best example of this type of cell is the epithelial cell, which covers the inner surface of the digestive system from mouth to the anus. In addition, regardless of whether they originate initially from the ectoderm, mesoderm, or endoderm, or differentiate into which epithelial tissue, despite the fact that the self-renewal features are automatically revealed, it shows that they never lose their multipotent stem or unipotent progenitor stem cell potentials [12]. Of course, the activities of epithelial cells are carried out by several mediators and signal pathways, including mediators. There is an important fact known of epithelial cells: these structures, regardless of the embryological eugenics they originated from or the type of epithelial tissue from which they differ, carry out their normal functions and use the same type of signaling pathways jointly [14,15,16,17]. One of the underlying causes of many epithelial disorders characterized by abnormal or uncontrolled proliferation, including cancers, is perhaps the deregulation of the factors controlling their growth. Although the possible causes and mechanisms leading to the deregulation are not yet fully elucidated, they make the subject much more interesting [18,19]. A number of questions can be asked in this context. How can cells deviate from this pathway into the excessive proliferation of cancer while following the periods of proliferation, maturation, differentiation, and elimination in the normal physiological state? Is it necessary to address overgrowth and cancer events as a sequence of events or as a result of different mechanisms? Regardless of the actual triggering and/or dissociating mechanisms, is not there a major intersection where these 2 realities intersect with each other during their respective processes? If so, can it be argued that this point of intersection is a matter of oxidative stress?

If we start with the last question, oxidative stress in itself is neither good nor bad; it is a normal phenomenon in the body. In other words, under normal conditions, many agents that we know causing oxidative stress (such as superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen) are generated as metabolic redox homeostasis and in vivo redox homeostasis [20,21].

If there is a problem, the source is not the formation or existence of free radicals that arise from exogenous origins (e.g., ultraviolet rays) or endogenous origins (at the cellular level, at which mitochondria are involved) but in an imbalance between free radicals and antioxidants in the body. If this imbalance passes to the second stage, i.e. oxidative stress due to excessive proliferation of cells in any tissue or organ, the capacity of the oxidation-reduction system of the body is exceeded. Thus, it would be almost impossible not to face abnormalities such as direct gene mutations or indirect carcinogenesis [22–24].

To ensure that the metabolism does not reach this stage, measures such as slowing or stopping cell division are activated. However, this applies only to certain healthy

cells. The answer produced against this process which intersects the hypoxia junction at the end, is the opposite in stem cells or cancer cells exhibiting stem-cell behavior, in a sense. These cell populations maintain cell proliferation in the face of hypoxia [25]. For most cell types, hypoxia induces increased cell proliferation since an increased number of cells, with a consequent increase in oxygen demand, would only exacerbate hypoxic stress. Healthy cells behave in this way but in cancer cells, certain cell populations maintain cell proliferation in the face of hypoxia. This is a common pathological hallmark of cancers, but it can also serve the physiological function of maintenance of stem cell populations that reside in a hypoxic niche [25,26]. If we continue with the example of abnormal cell proliferation, it is necessary to determine the relationship between oxidative stress and abnormal cell proliferation. The second thing to be done should be the evaluation of other factors involved in the initiation, promotion, and maintenance of this proliferative response. The third point, the most difficult one, is to try to prevent situations that cause abnormal proliferation, for example, oxidative stress, to protect the body from the consequences that will arise after the event, and, if it is possible, to cure this type of disease, for example, tumor-like masses or proliferative lesions by breaking the dilemma, which is known to have a causal relationship between abnormal proliferation and oxidative stress at that point. Research that will be done without consideration of specific features of epithelial cells and what can be the possible specific effects of rosemary on such tissues will not be entirely consistent with the facts. The explanation that overgrowth leads to oxidative stress and that imbalance due to increased oxidative stress that stops cell proliferation, seems to be the expected result of a rational logic but will not be of any scientific value.

In this context, our surprising findings have once again proved that the subject deserves attention. In this in vitro study, we applied 5 different doses of rosemary oil to the HaCaT and observed cell viability at 24, 48, and 72 h to contribute to the understanding of epithelial cell biology, which is known to be capable of exhibiting stem cell behavior. These findings might contribute to the prevention and/or treatment of certain diseases of epithelial origin that are characterized by excessive division (such as psoriasis, eczema, and ichthyosis of ectodermal origin) or of benign and malignant epithelial pathologies of exothermal origin that are very common in the gastrointestinal tract.

Our findings demonstrate that rosemary has antiproliferative effects in keratinocytes regardless of the dose and that the effect increases with the amount given. However, not only the dose but also time indicates that our results cannot be interpreted immediately and/or easily without considering some complex mechanisms. Further research is also needed to interpret the increase of

mean cell vitality at 74 h, after an initial decrease at 48 h. Additionally, we observed that the variance of cell vitality is decreasing with increasing doses of the rosemary oil extract, which resembles the effects of DMSO 20%. Thus, it can be considered that increasing dosage becomes similar to the toxic control.

Although the antiproliferative effect of *Rosmarinus officinalis* or its compounds has been successfully demonstrated in many studies, there is minimal information on the mechanism through which the plant exerts this effect, and only a few investigations mention the apoptosis pathway [27–29].

In 2016, carnosic acid, which is the most crucial bioactive component in *Rosmarinus officinalis*, was shown to have an apoptotic effect in CAKI cells through mitochondria-dependent caspase activation and to interfere with the signal transducer and activator of transcription (STAT3) signaling pathway via the generation of ROS [28].

It is known that apoptosis, which is the choice of a healthy metabolism to stop physiologically excessive cell proliferation that occurs for any reason, is the second method if necrosis fails. When each group of cells is exposed to excessive proliferation due to pathological reasons such as gene mutations, this time, if the immune and immune-endocrine system of the organism maintains its integrity, it will force these cells to undergo the apoptosis pathway. If this cannot be achieved, the option will be either necrosis or carcinogenesis.

As a result of the direct contact of the metabolism with both internal and external factors, epithelial cells, which are at a high risk of mutation, have to be renewed continuously due to hemostasis. This process, in one aspect (continuous regeneration), provides an advantage to the organism, while on the other hand, it carries the risk of being exposed to mutation for any reason. It is precisely this point where a dilemma arises. Given the functions mentioned above regarding the gastrointestinal tract, one would not exaggerate to assume a higher risk than other epithelial tissues. Moreover, in any possible defects of this system, the cells begin to regenerate abnormally, which in turn adversely affect digestion, and in later stages, lead to the collapse of the whole organism.

This study suggests that rosemary oil used in the cell culture inhibited excessive cell proliferation by the apoptotic pathway. We conclude that rosemary oil may help living organisms to have a healthy digestive system by stopping excessive cell proliferation via the apoptotic pathway in the gastrointestinal system, and thus may be used in both animal feed and human nutrition.

Furthermore, rosemary may contribute to the treatment of a number of diseases in the oral mucosa called "tumor-like masses," "proliferative lesions" or "benign soft tissue lesions," which may lead to nutritional disorders.

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