

ORIGINAL RESEARCH ARTICLE

Effects of oleuropein and hydroxytyrosol on brain renin-angiotensin system-regulating aminopeptidases in experimental glioma

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Abstract

Previous *in vitro* and *in vivo* studies have demonstrated that extra virgin olive oil polyphenols act against different pathologies, including several types of cancer such as glioma. Emerging therapies targeting the renin-angiotensin system (RAS) have shown therapeutic promise. Here we analyze the effects of the oral administration of oleuropein, hydroxytyrosol, and the mixture of both polyphenols in animals with gliomas to determine their effects on gliomagenesis and the regulatory proteolytic enzymes of the RAS of the aminopeptidase type, including the impact of sex differences. Gliomas were induced by the transplacental administration of N-ethyl-N-nitrosourea (ENU). Aminopeptidases were assayed fluorometrically using aminoacyl- β -naphthylamides as substrates. Kaplan–Meier survival curves revealed that these treatments significantly improve survival rates, with notable sex differences. In addition, their effects on tumor number and volume showed sex differences. Regarding the RAS-regulating aminopeptidases, our results support the idea of an increased effect of angiotensin III (AngIII) on untreated animals. In contrast, the findings with polyphenol treatments allow us to infer a decrease in AngIII and an increase of angiotensin 1 – 7, also with sex differences. A putative role on glucose uptake facilitation mediated by insulin-regulated aminopeptidase is also hypothesized. Our results demonstrated that local RAS significantly participates in gliomagenesis induced by transplacental ENU administration. In summary, orally administered extra virgin olive oil polyphenols, mainly hydroxytyrosol, showed differential effects against glioma, acting through the RAS-regulating aminopeptidase activities, and such differences were further compounded by sex disparity.

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1. Introduction

As a fundamental element of the Mediterranean diet, polyphenols in extra virgin olive oil have been demonstrated in recent research to exert suppressive effects against different pathologies, including cardiovascular diseases or different types of cancer.^{1–10} These minor components constitute approximately 1 – 2% of extra virgin olive oil and encompass over

230 distinct types of chemical compounds,^{1,11} suggests their high biological activity even at very low concentrations. Therefore, these polyphenols could be potentially useful for pharmacological interventions.

The primary polyphenols in extra virgin olive oil are alpha-tocopherol, oleuropein, and hydroxytyrosol. The latter is the most abundant, along with oleuropein, although the oleuropein content is highly dependent on the physiological development of the fruit.¹² Furthermore, oleuropein is highly hydrolyzed during its passage through the gastrointestinal tract^{1,13,14} so only a small amount could reach the systemic circulation. However, these compounds can cross the blood-brain barrier and reach the brain.¹⁵⁻¹⁷ Both compounds have been proposed as substances with antitumor effects in both *in vitro* and *in vivo* studies in several types of cancers, including glioma,¹⁸⁻²³ when used at relatively high concentrations in purified form.

Gliomas are the most common nervous system tumors, representing 23.41% of the primary brain tumors, with a higher rate in females than in males. Even today, gliomas have a poor prognosis, as a high portion of the glioma cases are determined as malignant, especially glioblastoma multiforme, which is associated with an estimated relative survival of only 35.8% in survivors 5 years after diagnosis.^{24,25}

Recently, the use of combination therapy of various drugs directed against the renin-angiotensin system (RAS) has been proposed against glioma,²⁶⁻³⁰ which, acting at the local level of the brain, presents promising results, based on the strong implication of this system in tumor development, especially angiotensin II (AngII) and angiotensin III (AngIII), which act at the level of their AT1 and AT2 receptors.³¹⁻³³ However, other components of the RAS, such as regulatory proteolytic enzymes of the aminopeptidase type, have also been related to tumor growth in gliomas, while there are minor alterations at the systemic level.²¹⁻²³

Previous studies of our laboratory using a heterotopic model of C6 glioma implanted in the subcutaneous region have shown that the antitumor action of both polyphenols is mainly due to the activity of hydroxytyrosol rather than oleuropein. This antitumor effect of hydroxytyrosol is prevalent at various levels, including the redox state, the anti-inflammatory capacity, and the capacity to modify the regulatory proteolytic enzymes of the RAS of the aminopeptidase type. In addition, many of these effects are dependent on sex; therefore, hormonal status is a parameter of great importance to take into account.^{21-23,34,35}

In the present study, we analyzed the effect of the oral administration of oleuropein, hydroxytyrosol, and the

mixture of both compounds in male and female animals with gliomas induced by the transplacental administration of N-ethyl-N-nitrosourea (ENU) to elucidate their effects on the regulatory proteolytic enzymes of the RAS of the aminopeptidase type at the brain versus plasma levels. We also investigated their relationship with the ability to act against tumors and the putative sex differences found in their actions.

2. Materials and methods

2.1. Animals, treatments, and sample preparation

Female ($n = 17$) and male ($n = 9$) Wistar rats were obtained from Harlan laboratories (Spain). The animals were maintained in a controlled environment under constant temperature (25°C) with a 12 h-light/12 h-dark cycle. Rats were housed in cages and given free access to standard laboratory rat food and drink. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). Protocols were approved by the Bioethical Committee of the University of Jaen (Reference number CVI09-4957M). Female Wistar rats weighing 200 – 250 g were caged overnight with males, and the day when the sperm was confirmed in vaginal smears was designated as day 1 of gestation. On day 18 of gestation, pregnant rats were injected i.v. with a single dose of ENU, 75 mg/kg body weight dissolved in saline solution. Female and male offspring from ENU-treated rats were utilized in these experiments. A total of 158 offspring were obtained. The offspring were naturally delivered and weaned at 22 days of age. At this time, males and females were housed separately and randomly divided into 4 groups. Group 1 (OLEU group, consisting of 34 animals: 20 females and 14 males) received oleuropein solution (50 mg/L) in drinking water. Group 2 (HTX group, consisting of 35 animals: 20 females and 15 males) received hydroxytyrosol solution (25 mg/L) in drinking water. Group 3 (MIX group, consisting of 35 animals: 19 females and 16 males) received oleuropein plus hydroxytyrosol solution (50 mg/L and 25 mg/L, respectively) in drinking water. Group 4 (Control group, 54 animals: 29 females and 25 males) received tap water. These doses were carefully chosen based on prior pre-clinical studies investigating the antitumor and bioactive effects of these polyphenols.^{21,23,34-36} Both oleuropein and hydroxytyrosol were obtained from Extrasynthese, CymitQuimica S.L., Barcelona, Spain. All other chemical compounds were obtained from Sigma-Aldrich, Madrid, Spain. All animals were allowed access to drink and food *ad libitum* and were kept under weekly observation for any sign of neurological or health problems. Surviving rats were killed after 30 weeks of treatment, and tumor and plasma samples were obtained.

2.2. Magnetic resonance imaging (MRI) analysis

Magnetic resonance images were acquired using 9.4 Tesla (Bruker Biospec, Ettlingen, Germany) at the Fundación IDICHUS (Santiago de Compostela, Spain). Twenty contiguous 1 mm thick slices for each animal were acquired using a T2-weighted spin-echo sequence. Tumor volume was calculated by analyzing the surface of the tumor on successive MRI slices multiplied by slice thickness. Images were analyzed and processed using Bruker's Paravision 5.1 software and Image-J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov/ij/>).

2.3. Plasma and tissue collection

Rats were anesthetized with equithesin (2 mL/kg body weight) by intraperitoneal injection and then shaved and sterilized with 10% povidone-iodine. Blood samples were obtained from the left cardiac ventricle, drawn into tubes with heparin as anticoagulant, allowed to clot and then centrifuged for 10 min at 3000× *g* to obtain the plasma, which was frozen and stored at −80°C until use. Samples of tumors were quickly removed and prepared for histopathological examination or frozen at −80°C until use.

2.4. Aminopeptidase activity assays

2.4.1. Aspartyl aminopeptidase (ASAP) activity assay

ASAP was measured fluorometrically using aspartyl-β-naphthylamide (AspNNAp) as the substrate. Briefly, 10 μL of each sample were incubated in triplicate for 30 min at 37°C with 100 μL of the substrate solution containing 100 μM AspNNAp, 1.3 μM ethylenediaminetetraacetic acid, and 2 mM MnCl₂ in 50 mM of phosphate buffer (pH 7.4).

2.4.2. Aminopeptidase A (APA) activity assay

APA activity was measured in the same way using glutamyl-β-naphthylamide (GluNNAp) as the substrate. Ten microliters of each sample were incubated in triplicate for 30 min at 37°C with 100 μL of the substrate solution containing 100 μM GluNNAp, 0.65 mM dithiothreitol (DTT), and 50 mM CaCl₂ in 50 mM of phosphate buffer (pH 7.4).

2.4.3. Aminopeptidase N (APN) activity assay

APN was measured fluorometrically using alanyl-β-naphthylamide (AlaNNAp) as substrate. Ten microliters of each sample were incubated in triplicate for 30 min at 37°C with 100 μL of the substrate solution containing 100 μM of AlaNNAp and 0.65 mM DTT in 50 mM phosphate buffer (pH 7.4).

2.4.4. Aminopeptidase B (APB) activity assay

APB was measured fluorometrically using arginyl-β-naphthylamide (ArgNNAp) as substrate. Ten microliters of each sample were incubated in triplicate for 30 min at 37°C with 100 μL of the substrate solution containing 100 μM of ArgNNAp and 0.65 mM DTT in 50 mM phosphate buffer (pH 7.4).

2.4.5. Insulin-regulated aminopeptidase (IRAP) activity assay

IRAP activity was measured fluorometrically using leucyl-β-naphthylamide (LeuNNAp) as substrate. Ten microliters of each sample were incubated in triplicate for 30 min at 37°C with 100 μL of the substrate solution containing 100 μM of LeuNNAp and 0.65 mM DTT in 50 mM phosphate buffer (pH 7.4).

All the reactions were stopped by adding 100 μL of 0.1 M acetate buffer (pH 4.2). The amount of β-naphthylamine released due to the enzymatic activities was measured fluorometrically at 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were also quantified in triplicate with the Bradford protein assay, using bovine serum albumin as standard. Specific enzyme activities were expressed as picomoles or nanomoles of the corresponding aminoacyl-β-naphthylamide hydrolyzed per min per mg of protein, determined using a standard curve prepared with the latter compound under corresponding assay conditions. The fluorogenic assay was linear with respect to the time of hydrolysis and protein content.

2.5. Statistical analysis

All values represent the mean ± standard error of the mean. The data were analyzed by multiple analyses of variance using GraphPad Prism V.8 software (GraphPad Prism version 8.4.3 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). Values of *p* < 0.05 were considered significant.

3. Results

3.1. Parameters of carcinogenesis

Figure 1 shows several examples of histopathological sections of tumors in untreated male and female rats and in male and female rats treated with oleuropein, hydroxytyrosol, or a mixture of oleuropein plus hydroxytyrosol.

Kaplan–Meier survival curves showed that male rats treated with oleuropein, hydroxytyrosol, or the mixture of oleuropein plus hydroxytyrosol had a significantly higher survival rate than untreated male rats (57.1%, 60%, and

Transplacental Ethyl-Nitrosourea-Induced Glioma

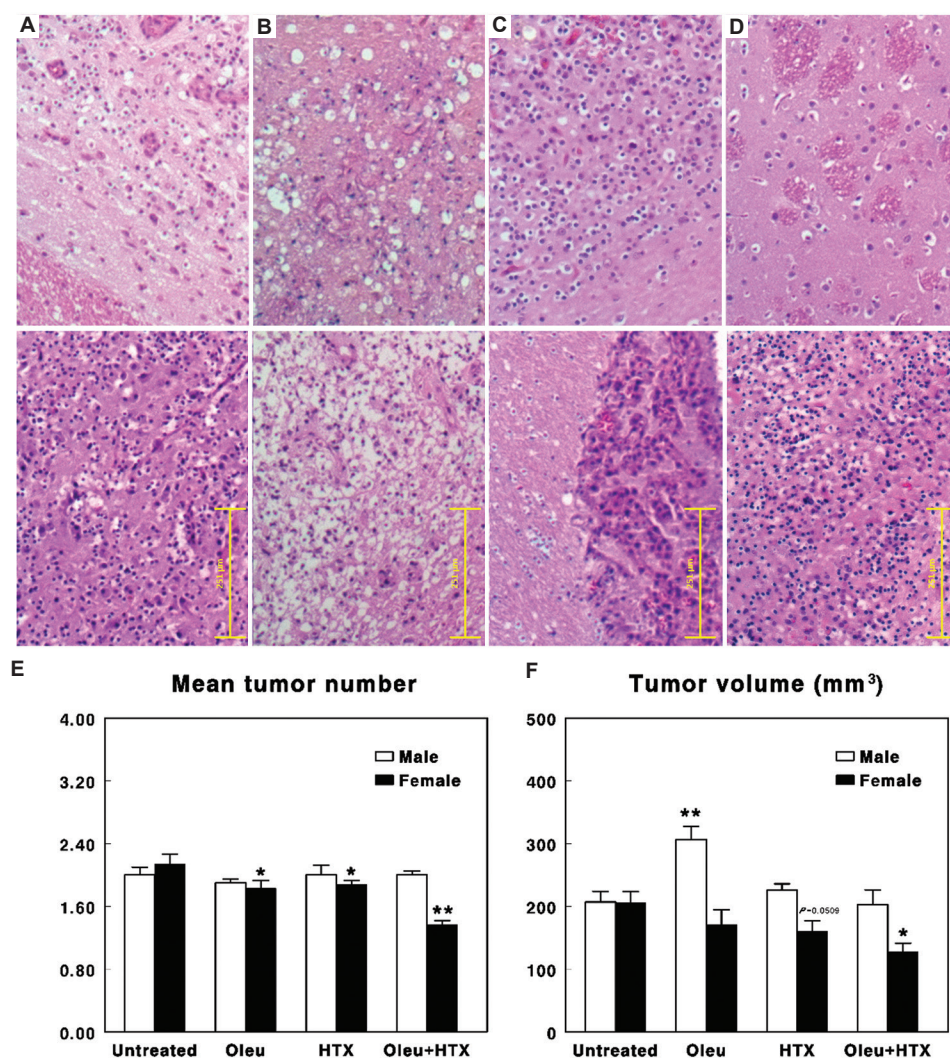


Figure 1. Histopathological studies showing representative specimens of the transplacental N-ethyl-N-nitrosourea -induced brain tumors in male (upper pane) and female (lower pane) rats untreated (A) or treated with oleuropein (B), hydroxytyrosol (C), or the mixture of oleuropein plus hydroxytyrosol (D) (microscopic view with H&E staining using $\times 10$ magnification). Mean tumor number per animal (E) and tumor volume (F) are presented for male and female animals. Volume values represent the mean expressed in $\text{mm}^3 \pm$ standard error of the mean ($n = 6$). * $p < 0.05$; ** $p < 0.01$ compared to untreated animals.

50%, respectively, versus 24.1% in 30-week survival, log-rank test, $p < 0.05$ in all cases). No significant differences in survival were found between treatments. In female rats, survival curves showed that the treatment with the mixture of oleuropein plus hydroxytyrosol promoted a significantly higher survival rate than that of untreated female rats (52.6% versus 24.1% in 30-week survival, log-rank test, $p < 0.05$) or with a borderline significance than the female rats given oleuropein or hydroxytyrosol alone (35% versus 24.1% in 30-week survival, log-rank test, $p = 0.0516$).

ENU-treated animals showed a tumor incidence, defined as the percentage of rats bearing at least one malignant tumor at sacrifice, of 100% in male and female rats. Furthermore, males showed a range from 1.9 ± 0.05 to 2.0 ± 0.12 tumors per animal, without significant differences after the treatments with oleuropein, hydroxytyrosol, or oleuropein plus hydroxytyrosol (Figure 1E). In contrast, untreated females showed a mean tumor number per animal of 2.14 ± 0.12 , whereas female rats treated with oleuropein or hydroxytyrosol showed a significant decrease in tumor number ($p < 0.05$ in both cases) by ≈ 1.2 -fold and ≈ 1.1 -fold, to 1.83 ± 0.1 and 1.88

± 0.05 tumors per animal, respectively. Finally, female rats treated with the mixture of oleuropein plus hydroxytyrosol showed the lowest mean number of tumors per animal (by ≈ 1.6 -fold, $p < 0.01$), measuring 1.37 ± 0.05 (Figure 1E). Regarding tumor volume, no significant difference was found between untreated male rats and after the treatment with hydroxytyrosol and the mixture of oleuropein and hydroxytyrosol, whose tumor volume measurements range from 203.3 ± 23.26 to 222.55 ± 10.44 mm³. However, males treated with oleuropein alone showed a significant increase in tumor volume by ≈ 1.5 -fold, to 306.32 ± 21.03 mm³ (Figure 1F). In female rats, a significant tumor volume decrease ($p < 0.05$) by ≈ 1.6 -fold, from 206.18 ± 17.52 mm³ to 127.43 ± 24.09 mm³, was found after the treatment with oleuropein plus hydroxytyrosol compared with untreated animals, whereas only a borderline significance was found in females treated with hydroxytyrosol, with values ranging from 206.18 ± 17.52 mm³ to 160.76 ± 16.55 mm³ ($p =$

0.0509). Oleuropein alone did not modify tumor volume in female animals with glioma.

3.2. Effects on RAS-regulating aminopeptidases

Figures 2 and 3 below present the circulating and tissue levels, respectively, of regulatory proteolytic enzymes of the RAS in both control and glioma-induced male and female animals, untreated or treated with oleuropein, hydroxytyrosol, or the mixture of both compounds orally administered in drinking water. Figure 2A shows that the circulating levels of ASAP were not modified between healthy animals and animals with tumors, and were only modified in male animals with gliomas administered with hydroxytyrosol or in female animals with gliomas administered with hydroxytyrosol or the mixture of oleuropein plus hydroxytyrosol, which is due to a preferential effect of hydroxytyrosol over oleuropein. In contrast, at the brain level (Figure 2B), untreated

RAS-Regulating Aminopeptidase Activities

Transplacental Ethyl-Nitrosourea-Induced Glioma

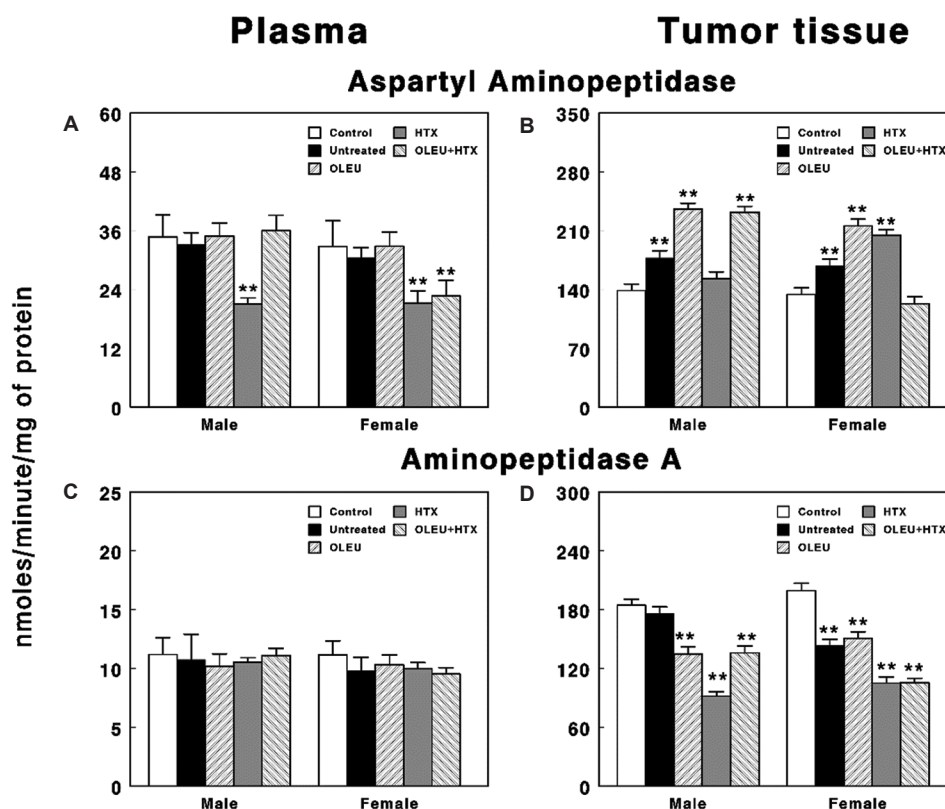


Figure 2. Circulating and tissue aspartyl aminopeptidase (A and B) and aminopeptidase A (C and D) specific activities in male and female healthy control rats versus rats with N-ethyl-N-nitrosourea-induced gliomas untreated or treated with oleuropein, hydroxytyrosol, or a mixture of oleuropein plus hydroxytyrosol, which were orally administered. Results are expressed in nanomoles of their corresponding aminoacyl- β -naphthylamide hydrolyzed per min and per mg of protein (mean \pm standard error of the mean; $n = 6$; ** $p < 0.01$ compared to control animals).

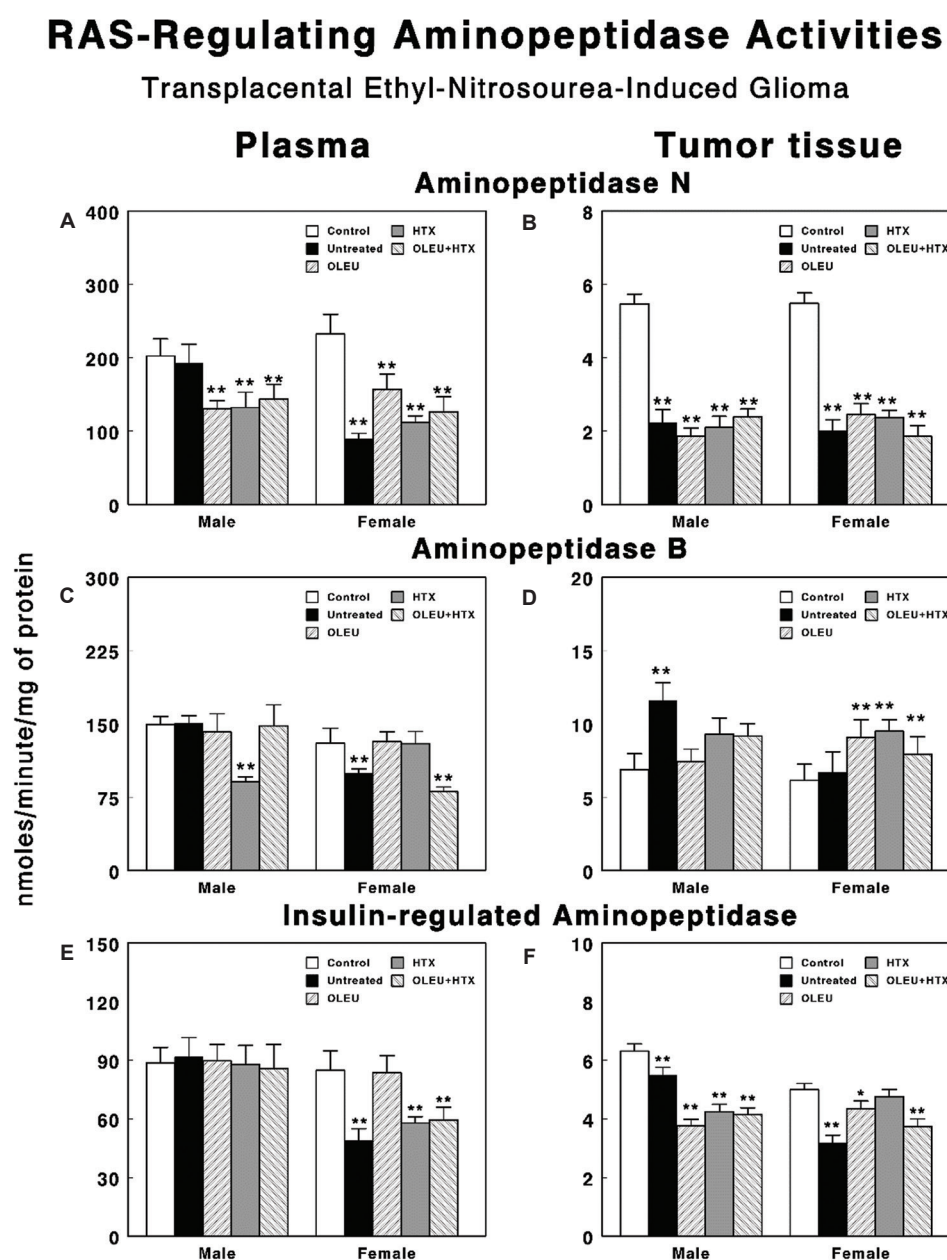


Figure 3. Circulating and tissue aminopeptidase N (A and B), aminopeptidase B (C and D), and insulin-regulated aminopeptidase (E and F) specific activities in male and female healthy control rats versus rats with N-ethyl-N-nitrosourea-induced gliomas untreated or treated with orally administered oleuropein, hydroxytyrosol, or the mixture of oleuropein plus hydroxytyrosol. Results are expressed in nanomoles of their corresponding aminoacyl- β -naphthylamide hydrolyzed per min and per mg of protein (mean \pm standard error of the mean; $n = 6$; * $p < 0.05$; ** $p < 0.01$ compared to control animals).

animals, and both males and females, showed a significant increase in ASAP activity. This activity increased even more in male animals treated with oleuropein or the mixture of oleuropein plus hydroxytyrosol, but it was not modified in the animals to which only hydroxytyrosol was administered. In this case, it would imply a preferential effect of oleuropein. In the case of females, the ASAP activity increased even more in those animals treated with

oleuropein and hydroxytyrosol but not with the mixture of both compounds. Consequently, these results imply that the effects of both compounds interfere in their actions on the activity of this enzyme.

No significant differences in the activity of the circulating APA were found either in male or female animals due to the administration of ENU or with the oral administration of the studied substances (Figure 2C).

However, at the tissue level, no significant differences in APA activity were found in untreated male animals compared with their healthy controls; although the administration of both oleuropein and hydroxytyrosol or a mixture of both induced a significant decrease in APA activity, mainly with the administration of hydroxytyrosol, which is due to a preferential effect of hydroxytyrosol over oleuropein. In the case of females, untreated animals with gliomas showed a significant decrease in APA activity, whereas animals treated with oleuropein maintained this significant decrease. However, the strongest significant lowering effects on APA were found with the administration of hydroxytyrosol or the mixture of both oleuropein plus hydroxytyrosol, alongside notable observation of sex differences in these results (Figure 2D).

In the case of APN, at the circulating level, we did not find in males significant differences between control animals and animals with ENU-induced gliomas, but APN activity was significantly decreased in females. However, in male animals, the administration of oleuropein, hydroxytyrosol, or a mixture of both compounds causes a significant decrease in APN activity (Figure 3A). In females, APN activity remains low regardless of the administration of the different compounds in isolation or combination. A similar pattern is found at the level of brain tissue, except in untreated male animals with ENU-induced gliomas showing a significant decrease in this enzyme activity (Figure 3B). However, the administration of oleuropein, hydroxytyrosol, or the mixture of both compounds promotes lower levels of APN activity again compared with healthy control animals.

Different results were obtained for APB activity. At the circulating level, no significant differences appear in males as a consequence of the induction of gliomas with ENU, and only the administration of hydroxytyrosol significantly decreased APB activity. On the contrary, in female animals, the induction of gliomas significantly decreased the circulating activity of APB, which remained diminished only in those animals to which the mixture of hydroxytyrosol plus oleuropein was orally administered. When these substances were administered alone, the values found were similar to those of healthy control animals (Figure 3C). The opposite was observed regarding the behavior at the level of brain tissue. Male animals with ENU-induced tumors showed levels of APB activity higher than their healthy controls, and the administration of oleuropein, hydroxytyrosol, or the mixture of both compounds returned these levels to their normal values. On the contrary, in female animals, the induction of tumors by ENU did not modify this enzymatic activity, while the administration of oleuropein, hydroxytyrosol,

or the mixture of both compounds significantly increased APB activity in all cases (Figure 3D).

Finally, it should be noted that we did not find any alteration in the circulating levels of IRAP in male animals, neither as a consequence of the induction of tumors by ENU nor by the oral administration of oleuropein, hydroxytyrosol, or the mixture of both compounds. However, circulating IRAP activity in female animals was significantly decreased due to the induction of tumors by ENU, and only the administration of hydroxytyrosol or the mixture of oleuropein plus hydroxytyrosol returned this activity to control levels, which seems to be especially due to the administration of hydroxytyrosol (Figure 3E). In the case of brain tissue, both male and female untreated animals with ENU-induced gliomas showed a significant decrease in IRAP activity. In males, this activity was even lower when oleuropein, hydroxytyrosol, or a mixture of both compounds was administered. However, in female animals, the administration of hydroxytyrosol reverted this enzymatic activity to its control values, which was not achieved either with the administration of oleuropein or with the administration of the oleuropein plus hydroxytyrosol mixture. This result suggests a reversal effect under some circumstances when both components are administered together (Figure 3F).

4. Discussion

The RAS has emerged in recent years as a potentially useful pharmacological target in the treatment of glioma.²⁶⁻³⁰ In this way, it is well described that AngII stimulates tumor neovascularization³⁷ and overexpression of angiotensin-converting enzyme (ACE) and AT1 is associated with tumor growth metastasis and progression.³⁸⁻⁴¹ In addition, interesting results have been obtained in patients with cancer who were already being treated well with ACE inhibitors and with angiotensin receptor blockers (ARBs). Thus, ACE inhibitors seem to reduce cancer incidence or, at least, protect against it by reducing cell proliferation and migration, inflammation, and angiogenesis.^{42,43} Furthermore, these compounds inhibit the cellular matrix metalloprotease activity and reduce the expression of vascular endothelial growth factor.^{43,44} ARBs have also demonstrated their ability to decrease tumoral volume, mitotic index, cell proliferation, and the number of capillary vessels.⁴⁵ In fact, in recent years, the use of combination therapy of these and other drugs against glioma has been proposed, which seems to be giving encouraging results.³¹⁻³³ Therefore, it is of great interest to search for compounds that somehow influence the RAS because they can also be effective against glioma. It is even better if they are compounds of natural origin or part of food because they can also have a preventive nature. In this

sense, oleuropein and hydroxytyrosol, key components of extra virgin olive oil, a fundamental element of the Mediterranean diet, have been demonstrated to provide health benefits across various conditions, mainly focused not only in cardiovascular and circulatory diseases but also in cancer. Thus, we analyzed in this study the effect of oral administration of oleuropein and hydroxytyrosol alone or in combination on cerebral gliomas induced by transplacental administration of ENU, and their influence on the enzymatic activity of several of the proteolytic regulatory enzymes of aminopeptidase type of the circulating and local RAS. This analysis was conducted in both male and female animals because the sex differences of those regulatory enzymes have been well demonstrated,⁴⁶ with the behavior of the tumoral process varies with sex.³⁶ Despite the important role of regulatory aminopeptidases in the RAS, they have received much less attention as potential therapeutic targets against glioma. Interestingly, they are clearly considered cell-surface peptidases involved in controlling cell proliferation and differentiation by modulating the access of peptides to their membrane receptors. Their alteration may contribute to neoplastic transformation or cell progression.⁴⁷⁻⁴⁹

4.1. Effects of phenolic compounds on survival and carcinogenesis parameters

We demonstrated here that the administration of oleuropein, hydroxytyrosol, or a mixture of both polyphenols showed differential effects on the survival of animals with ENU-induced gliomas and sex differences. Thus, in male animals, the administration of either of the two polyphenols alone or in combination increased the survival of the animals compared to the untreated ones. In females, the effect was not so powerful; only the treatment with the mixture of oleuropein plus hydroxytyrosol showed significant differences compared to untreated animals, although animals that were given hydroxytyrosol, or to a lesser degree, oleuropein, also showed this trend. Perhaps these borderline sex differences could be better defined in a study that used an even larger number of animals than the size used in the present study. However, various authors have found sex differences in various indicators such as the magnitude of inflammation and edema, cellularity, or microvasculature, suggesting higher values in male animals. Another element, such as a greater breakdown of the blood-brain barrier, also occurs to a greater extent in male animals. In general, these and other data suggest more malignant and aggressive tumors in male animals.⁵⁰ Differences between men and women have also been observed in human patients.⁵¹ Therefore, the sex differences found here are possibly attributed to the different mechanisms affected by the action of the different polyphenols used.

Concerning the parameters of carcinogenesis, our results also showed sex differences in the effects of oleuropein, hydroxytyrosol, and the mixture of both polyphenols on the number and volume of tumors. None of the polyphenols, alone or in combination, modified the number of tumors in male animals, but the tumor number in female animals changed following the intake of hydroxytyrosol or the mixture of both polyphenols. Similarly, tumor volume was not affected in males, except for those receiving oleuropein treatments, which significantly increased tumor volume, while a decrease in tumor volume occurred in female animals.

The increase caused by oleuropein in male animals has already been described previously,³⁵ but the exact mechanism by which it occurs remains unresolved. However, this increase in tumor volume does not appear to be related to the survival of the animals and could be related to changes in cell cycle mechanisms or activation mechanisms of cell division. These effects could also be due to the powerful antioxidant capacity of oleuropein,⁵² which would be able to prevent DNA damage in the tumor cells.⁵³ In any case, these effects of oleuropein when administered alone were absent when oleuropein is administered in combination with hydroxytyrosol, which shows that it is an unknown but highly specific effect. It has even been suggested that it could be due to the inhibition of immune response against tumors, the possible structural similarity of oleuropein with growth factors or the interaction with its receptors, or its ability to inhibit cell death induced by free radicals.³⁵ We found a decrease in the number of tumors and tumor size in female animals treated with hydroxytyrosol or a mixture of both polyphenols, with the latter probably attributed to the action of hydroxytyrosol rather than the action of oleuropein. The antiproliferative effect of hydroxytyrosol at various levels is well known,^{54,55} but it seems to be highly related to hormonal status. It must be considered that the aromatic ring present in these compounds is a common characteristic with estradiol, which suggests a possible mechanism of action of these compounds correlated with their ability to compete with estrogen receptors and, thus, with the multiple signaling pathways mediated by these receptors.¹ Overall, the effects described here are limited by the dose of polyphenols actually ingested by animals because it is well known that the *in vivo* effects of these compounds depend on the concentration used.

4.2. Effects of phenolic compounds on RAS-regulating aminopeptidase activities

Our work described the changes in the activity of several aminopeptidases regulating the RAS and the effects of the subcutaneous injection of oleuropein, hydroxytyrosol, and the mixture of both phenolic compounds in tumor tissue in

a heterotopic model of subcutaneous C6 glioma. To avoid the limitations of this model, we obtained data from the animal model induced by ENU and evaluated the effects of the oral administration of these compounds alone or in combination on RAS-regulating aminopeptidase activities in both tumor tissue and plasma.

First, it is shown that the alterations in the circulating proteolytic activity in untreated animals are limited to the activities of APN, APB, and IRAP, but with an important sex difference because they only appear altered in females. However, the activities of the circulating ASAP and APA showed no variation in either male or female untreated animals. We have previously described the sex differences in the development of gliomas in the ENU-induced animal model, where the alterations in males and females in the redox systems are also different.³⁶ Therefore, it is obvious that the hormonal status decisively influences the progress of events occurring during the development of cerebral gliomas, and the circulating aminopeptidase activities could reflect the changes occurring at the brain level. These sex differences are also visible after the treatments with oleuropein, hydroxytyrosol, or a mixture of both compounds. Thus, in males, hydroxytyrosol alone modified ASAP and APB, whereas all tested compounds modified APN and had no effect on APA and IRAP. On the contrary, in females, the treatments had no effect on APA, but oleuropein, hydroxytyrosol, or the mixture of both polyphenols modified ASAP, APN, APB, and IRAP at different extents, either maintaining the differences found in untreated animals or restoring the values to control levels. At this point, it is worth noting that there is not always a summation effect of the mixture of oleuropein plus hydroxytyrosol treatment compared with the treatments with a single component. These may indicate that different mechanisms of action occur due to oleuropein or hydroxytyrosol, and probably not only related to their antioxidant properties, as previously described.³⁵

Regarding the enzymatic activity at the tissue level, significant differences appeared in most of the aminopeptidase activities analyzed in untreated animals, and there are also sex differences in some of them. Thus, ASAP, APN, and IRAP showed the same behavior in male and female animals. In the case of ASAP, there was a significant increase in enzymatic activity, while APN and IRAP showed a significant decrease. However, the activities of APA and APB showed sex differences, with APA activity significantly decreased in female animals while APB significantly increased in males. The biological properties of angiotensins are associated with the hormonal status and invasive potential of cancer cells.⁵⁶ Therefore, hormonal influence on aminopeptidase activities is also

plausible. In this regard, we have previously described several sex differences in aminopeptidase activities.⁴⁶ Moreover, the treatments with oleuropein, hydroxytyrosol, and the mixture of both compounds also modified the aminopeptidase activities in different complex ways depending on the treatment and sex differences. As stated before, these effects maintain the differences found compared to untreated animals, exacerbate them, or return the values to control levels. Similarly, the mixture of oleuropein and hydroxytyrosol does not always lead to a summation effect as compared to the individual treatments, suggesting a differential role of both compounds not only related to their antioxidant properties.

Taken as a whole, and considering the general scheme of the RAS cascade, the data related to tumor tissue obtained here regarding ASAP and APA allow us to infer the nuances in three possible routes between males and females, but with the same final outcome. First, in untreated animals, the activation of ASAP would promote, in both males and females, the formation of angiotensin 2 – 10 from angiotensinogen, providing the appropriate precursor for the formation of AngIII by ACE. Here, the administration of oleuropein and the mixture of both polyphenols in males or oleuropein and hydroxytyrosol in females increased ASAP activity. In contrast, hydroxytyrosol in males and the mixture of both polyphenols in females return the values to control levels, avoiding the accumulation of AngIII.

Second, the cleavage of AngII by ASAP is enhanced to form AngIII again in untreated animals. Similarly, only hydroxytyrosol in males and the mixture of both polyphenols in females return the values to control levels, avoiding the accumulation of AngIII. A main role of AngIII in carcinogenesis has been reported, as previously proposed^{34,57} or described for other authors.⁵⁸ It has been described that similar to AngII, AngIII activates cell growth. The proliferative signal transduction of AngIII seems to occur through MAPK phosphorylation, which is also similar for AngII.⁵⁹ AngIII appears to bind to the AT1 receptor in a concentration-dependent manner, resulting in cell proliferation.⁵⁸ Interestingly, AngIII was shown to be several times more effective in the brain than AngII in exerting key cellular responses. This enhanced efficacy manifests through multiple mechanisms, including differential metabolic stability, receptor activation dynamics, and downstream signaling pathways.⁶⁰

A conflicting aspect is the decrease in APA activity found in untreated females, which is also enhanced by the administration of hydroxytyrosol and the mixture of both polyphenols or the decrease induced in males with all the treatments administered. These data suggest that these compounds would prevent the formation of AngIII from

AngII. It has been described that APA was upregulated and enzymatically active in blood vessels of human tumors but was not detected in normal blood vessels.⁶¹ APA may be involved in the neovascularization of tumor development. However, it has been described in tumoral cells; therefore, this enzyme may play a regulatory role in both neoplastic transformation and disease progression.

Third, ASAP and APA could also participate in the inactivation of angiotensin 1 – 7 in favor of its metabolite angiotensin 2 – 7 in untreated animals. Similarly, an inhibition in the catabolism of angiotensin 1 – 7 could be promoted by the polyphenols intake. It has been demonstrated that angiotensin 1 – 7 opposes to AT1 activation by AngII/AngIII acting through the Mas receptor. This angiotensin 1 – 7/Mas axis plays an antitumorigenesis role, decreasing growth and invasiveness in some kinds of cancer. *In vitro*, angiotensin 1 – 7/Mas signaling inhibition increased cell invasion and proliferation in glioblastoma multiforme.^{62–64} However, the conflict that arose in females between ASAP and APA activity would again be present at this cascade level because, as mentioned above, the first activity tends to increase while the second is inhibited. In this regard, an additional role for APA not related to neovascularization but related to the permeabilization of the blood-brain barrier has been proposed.^{65,66}

In the same way, the decrease in APN and IRAP activities found in both male and female untreated animals also allows us to infer a decreased catabolism of AngIII toward angiotensin IV (AT4), which would again enhance AngIII effects. However, none of the treatments administered manages to reverse these effects, except hydroxytyrosol in the case of females on IRAP activity, although as we will see later, this could be related to other processes in which this enzyme is involved. It is worth noting that the APB activity is significantly increased in untreated male animals but unchanged in untreated female animals, which could indicate a hormone-dependent alternative function to the conversion of AngIII to AngIV as mentioned above, and that should be further investigated. However, a limited action of APB on AngIII has been described,⁶⁷ also supporting the idea that APB is involved in generating additional active peptides by cleaving extended amino-terminal arginine and lysine residues. However, all treatments decreased APB activity in males to its normal levels, while in female animals, all treatments caused an increase in its activity, giving APB activity a significant role in the development of glioma, which should be further analyzed.

As stated above, an additional fact is to consider that IRAP also acts as a receptor for AT4, so that the alteration of the enzymatic activity, which would promote a lower

amount of AT4, could also reflect a lower activation of the AT4 receptor in untreated animals, except for females treated with hydroxytyrosol. In addition, the changes that we found in the circulating activity of APN, APB, and IRAP in untreated female animals exclusively would also be in favor of suggesting an increased activity of AngIII, through regulatory mechanisms that would be different in males and females, thus suggesting a hormonal dependence. We should bear in mind that our group has already described findings of sex differences in tumor development in glioma, as stated above.

Finally, IRAP is also related to the insulin-responsive glucose transporter (GLUT) type 4 (GLUT4). IRAP codistributes with GLUT4 in specialized vesicles, where it plays a role in the tethering and/or trafficking of these vesicles.^{68–70} The GLUT was thought to facilitate glucose uptake into cells. AT4 enhanced this activity-dependent glucose uptake in some brain regions, but not in others, supporting that the modulation of glucose uptake by AT4 is region-specific and is critically dependent on a high degree of colocalization between IRAP and GLUT4. The expression of facilitative GLUT isoforms in human astrocytic tumors was examined. The results showed that a biopsied glioblastoma expressed the genes of *GLUT1*, *GLUT3*, and *GLUT4* glucose transporter. Northern blot analysis of total RNA from a biopsied glioblastoma showed the transcripts of only *GLUT1* and *GLUT3*, suggesting that the expression of insulin-responsive glucose transporter *GLUT4* mRNA is relatively low. These results suggest that the facilitative glucose transport may be altered in astrocytic tumor cells and thus display a significant change in glucose metabolism,⁷¹ which is consistent with our results. In addition, the use of GLUT inhibitors enhances the action of bis-chloroethyl nitrosourea and temozolomide against high-grade glioma,⁷² also supporting an unexplored role of IRAP in glioma treatment. Treatment with the different polyphenols, alone or in combination, would be consistent with this hypothesis in male animals, where the IRAP activity would be even lower than in untreated animals. In female animals, the behavior would be different because the treatments tend to increase the enzymatic activity to values similar to those of healthy controls. In any case, the existence of sex differences in the behavior of the regulatory proteolytic enzymes and the effect of the treatments is evident.

5. Conclusions

Gliomagenesis induced by transplacental administration of ENU shows a substantial involvement of the local RAS in both male and female animals with significant sex differences. Even in female animals, some of these alterations are reflected at the level of the circulating RAS.

The administration of oleuropein and hydroxytyrosol has differential effects, depending on the sex, which is capable of promoting a smaller number of tumors in female animals only. In addition, in females, a summation effect of oleuropein and hydroxytyrosol seems to be found when they are administered together. Similarly, only hydroxytyrosol is clearly able to significantly reduce tumor volume in females, a phenomenon that is not so clear in males. In these male animals, even oleuropein causes a significant increase in tumor volume, a phenomenon that we had already observed in the glioma animal model due to the subcutaneous implantation of C6 cells, and which is probably due to the existence of a redox state that promotes tumor growth in these animals.³⁵ Similarly, RAS regulatory proteolytic enzymes are also a potential target for treating these types of tumors. Their pharmacological manipulation can be as interesting as that of other better-investigated RAS elements, such as receptor blockers or ACE inhibitors, either alone or in combination with other drugs. This constitutes an innovative field of study because proteolytic enzymes of RAS have not been given sufficient attention in the treatment of tumors, considering the limitations of the multiple available targets in the complex cascade of RAS regulation.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Experimental protocols in animals were approved by the University of Jaen Bioethical Committee (reference code CVI09-4957M).

Consent for publication

Not applicable.

Availability of data

Data are available on request to the corresponding author.

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