



# The Protective Effect of Taurine and Curcumin on Autophagy-Related Genes in the Oocytes of the Mouse Treated with Acrylamide

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## Abstract

**Background:** Autophagy is also essential for both male and female infertility since it controls the development of germ cells and reproductive organs. This study aimed to investigate the effects of taurine and curcumin on the expression of genes related to autophagy in acrylamide-treated mice.

**Methods:** In 2022, this experimental study was conducted at the Shiraz University of Medical Sciences, Stem Cells Technology Research Centre. Forty-eight mice were randomly assigned to eight groups (control, curcumin 200 mg/Kg, taurine 150 mg/Kg, acrylamide 50 mg/Kg, acrylamide+curcumin 100 mg/Kg, acrylamide+curcumin 200, acrylamide+taurine 75 mg/Kg, acrylamide+taurine 150 mg/Kg). Finally, oocyte characteristics and gene expression were determined in each group using one-way analysis of variance (ANOVA) by SPSS 25 and GraphPad 9, respectively.  $P < 0.05$  was conducted statistically significant.

**Results:** A significant decrease was observed in several oocytes in the acrylamide group compared to the control group ( $P < 0.001$ ). The expression levels of light chain 3 (*LC3*), autophagy-related gene (*ATG12*, *ATG5*, and *Beclin1*) significantly increased in the acrylamide compared to the control group. A significant increase in the number of oocytes was observed in the taurine group compared to the control. The expression levels of *LC3*, *ATG12*, *ATG5*, and *Beclin1* significantly decreased in the acrylamide+taurine (150 mg/Kg) compared to the acrylamide group.

**Conclusion:** The acrylamide negatively impacts oocyte viability and causes the higher expression of autophagy-related genes. Taurine may encourage the fusion of autophagosomes with lysosomes by removing autophagic obstruction, potentially accelerating autophagy and protecting against oxidative stress. Taurine is more effective than curcumin at reducing the harmful effects of acrylamide. As a result, taurine can be proposed as a potential treatment drug for acrylamide-induced infertility.

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**Keywords** • Acrylamide • Oocyte • Mitochondria • Autophagy • Taurin • Curcumin

## What's Known

- Acrylamide exerts its effects through the induction of oxidative stress, which results in autophagy. High levels of oxidative stress can cause DNA damage and lipid peroxidation, which critically affects the etiology of infertility.

## What's New

- Acrylamide negatively impacts oocyte viability and causes autophagy-related genes to be expressed at higher levels. It was shown that taurine was more effective than curcumin at reducing the harmful effects of acrylamide. As a result, curcumin should be used with greater caution during the first trimester of pregnancy.

## Introduction

Acrylamide (ACR) is a widely used chemical in food packaging, textile production, adhesives, contact lenses, wastewater treatment, cosmetics (skin creams), drinking water, and tobacco and plastic industries. The primary dietary sources

of acrylamide include potato products, grain and cereal products, dried products, roasted nuts, and coffee.<sup>1</sup> Acrylamide, by disrupting the sex hormone system, causes ovarian tissue damage and has detrimental effects on fertility. Acrylamide exerts its effects through the induction of oxidative stress, which results in autophagy.<sup>2</sup> Treatment of rats with high doses of ACR increased the expression of autophagy-related genes such as autophagy-related 12 (ATG12), ATG5, and light chain 3 (LC-3).<sup>3, 4</sup> As the main cellular mechanism in many organs' reactions to acrylamide exposure, autophagy is essential for the regulation of early reproductive processes in the reproductive system, including gamete maturation and fertilization.<sup>5</sup> The process of autophagy generates a catabolic pathway that uses the lysosome to break down proteins and cellular organelles. The autophagy of intracellular proteins and organelles saves energy, allowing the body to adapt to changing conditions.<sup>6</sup>

The development of autophagosomes and signaling pathways depends on autophagy-related genes. *Beclin-1* is a known gene in the autophagy pathway that promotes the autophagosome's nucleation stage and links other relevant proteins to autophagy. Along with *Atg16L* and *Atg12*, *Atg5* is involved in forming membrane curvature and autophagosome maturation.<sup>7</sup>

High levels of oxidative stress can cause DNA damage and lipid peroxidation, which critically affects the etiology of infertility.<sup>8</sup> Studies demonstrated that using antioxidants could be a suitable solution for mitigating the adverse effects of reactive oxygen species (ROS).<sup>9, 10</sup>

Taurine (Tau), a derivative of the amino acid cysteine, is the most prevalent non-protein amino acid in the human body. Numerous investigations have demonstrated that Tau's antioxidative, anti-cell death and anti-inflammatory properties contribute to its ability to prevent xenobiotic-induced reproductive damage. The precise targets of Tau throughout the intracellular routes are linked to mitochondrial functionality. Furthermore, exogenous Tau supplementation is beneficial for several disorders.<sup>11, 12</sup>

Tau has been shown to enhance energy metabolism and mitochondrial function significantly.<sup>13</sup> Additionally, Tau may significantly reduce ROS produced by mitochondria.<sup>14</sup> It is also clear that mitochondria emit mediators of cell death and repress mitochondria-mediated cell death.<sup>15</sup>

Curcumin, another antioxidant molecule, is the main active ingredient of turmeric. It has antioxidant and anti-inflammatory properties;

however, conflicting studies persist on its effects on ovarian tissue.<sup>16, 17</sup> Curcumin has been thoroughly investigated and is thought to be an anti-cancer agent for several types of cancer, including skin, stomach, liver, lung, and colon cancers. It is also an active component with anti-inflammatory, antioxidation, antibacterial, antiviral, and antifungal properties.<sup>18</sup> Furthermore, studies in biology and medicine have demonstrated that curcumin is a safe and efficient treatment for cancer, particularly stomach cancer. However, due to curcumin's instability in alkaline conditions and limited solubility in water and acidic pH, it has not been widely employed in oral medications.<sup>18, 19</sup> Taurine and curcumin can both be considered therapeutic candidates for treating autophagy-related female infertility. As there have been few studies on the effects of ACR on the expression of genes involved in the autophagy process in mouse oocytes, the purpose of this study was to examine the effects of taurine and curcumin on the expression of autophagy-related genes in the mouse oocytes under acrylamide treatment.

## Materials and Methods

This experimental study was conducted in 2022 at Shiraz University of Medical Sciences (Shiraz, Iran) Stem Cells Technology Research Center. The procedures of this experiment were approved by the Research Ethics Committees of the Islamic Azad University Shiraz Branch (IR. IAU.SHIRAZ.REC.1402.041).

### Animals

The experimental animals used in this study were 48 adult female mice from the Naval Medical Research Institute, with an average weight of 30-35 g, aged 6-8-week-old. They were selected randomly and kept under standard conditions for 2 weeks before treatment to ensure adaptation.<sup>20</sup> The animal room was provided with a 12-hour light-dark cycle, and a controlled temperature (20±2 °C). *Ad libitum* access to water and standard rat chow (Behparvar®, Tehran, Iran) was provided.

### Experimental Design

The mice were grouped equally into 8 (n=6), and treated as follows:

Group 1 (Control) received physiological serum, and Group 2, 3, and 4 were treated with 200 mg/Kg curcumin (Merck-German, s7359554), 150 mg/Kg taurine (Sigma, T0625-100G, UK), and 50 mg/Kg acrylamide (Merck-German, 79-06-1), respectively. Group 5 and 6 received acrylamide+100 mg/Kg curcumin and

acrylamide+200 mg/Kg, respectively. Groups 7 and 8 received acrylamide+75mg/Kg taurine and acrylamide+150 mg/Kg taurine, respectively. The materials, including physiological serum, acrylamide, taurine, and curcumin, were administered daily for 35 days via oral gavage using a gastric needle. It is worth noting that taurine and curcumin were administered one hour before acrylamide administration.

#### Oocyte Collection

Female mice were super-ovulated with an intraperitoneal (i.p.) injection of 10 IU PMSG (Gonaser, HIPRA, Spain), followed by an i.p. injection of 10 IU hCG (Organon, Oss, Netherlands) on the last administration day. Between 10-12 hours post-HCG injection, the mice were euthanized by cervical dislocation, and then the oocytes were retrieved from the ampulla region of the oviduct. After briefly incubating the oocytes in 1 mg/mL hyaluronidase (4272, Sigma, UK), the cumulus cells were removed. Then, the cumulus-free oocytes were washed in preincubated fresh M2 (5910, Sigma, UK) media three times.<sup>21</sup> Following the oocyte collection, the effects of acrylamide, taurine, and curcumin on oocytes in different groups were assessed by inverted microscopy (Nikon, Japan), and autophagy gene expression was evaluated by real-time polymerase chain reaction (real-time PCR).

#### Evaluation of the Level of Autophagy Gene Expression

Initially, total RNA was extracted from the oocytes (n=20 in each group) using TRIzol reagent (15-596-026 Invitrogen, USA).<sup>22</sup> Next, the total concentration of RNA was determined using a NanoDrop (NanoDrop™, Thermo Fisher Scientific, Wilmington, DE, USA). Then, the first strand of cDNA was synthesized using a Takara kit (Prime Script™ RT reagent Kit, Takara: RR037B, AUS) according to the manufacturer's instructions. All samples were

prepared for quantitative real-time PCR (qRT-PCR) conditions. The expression of *Atg-5*, *Atg12*, *Lc3*, and *Beclin-1* genes were evaluated by Sybr Green (SG) Premix EX Taq™ II (RR650A) using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA).<sup>23</sup> The primers were designed using the gene bank Primer-BLAST online program (table 1). The standard curve method was used to estimate the efficiency of a real-time PCR reaction amplification (data is not shown here) and then examine the expression of each gene in the samples.  $\beta$ -actin, as an internal control, was used to normalize the amplification signals of samples. All experiments were performed in triplicate, and relative expression was calculated using the  $2^{-\Delta\Delta Ct}$  method, which compared it with the corresponding control group.

#### Statistical Analysis

Version 23 of the SPSS software (Armonk, NY: IBM Corp, USA) was used for statistical analysis. First, all data were normalized, then One-way analysis of variance (ANOVA) Tukey's multiple comparison test was used to examine the data in the desired groups by GraphPad Prism software version 9 (GraphPad Software Inc. La Jolla, California, USA). Data were considered significant at a level of  $P < 0.05$ .

## Results

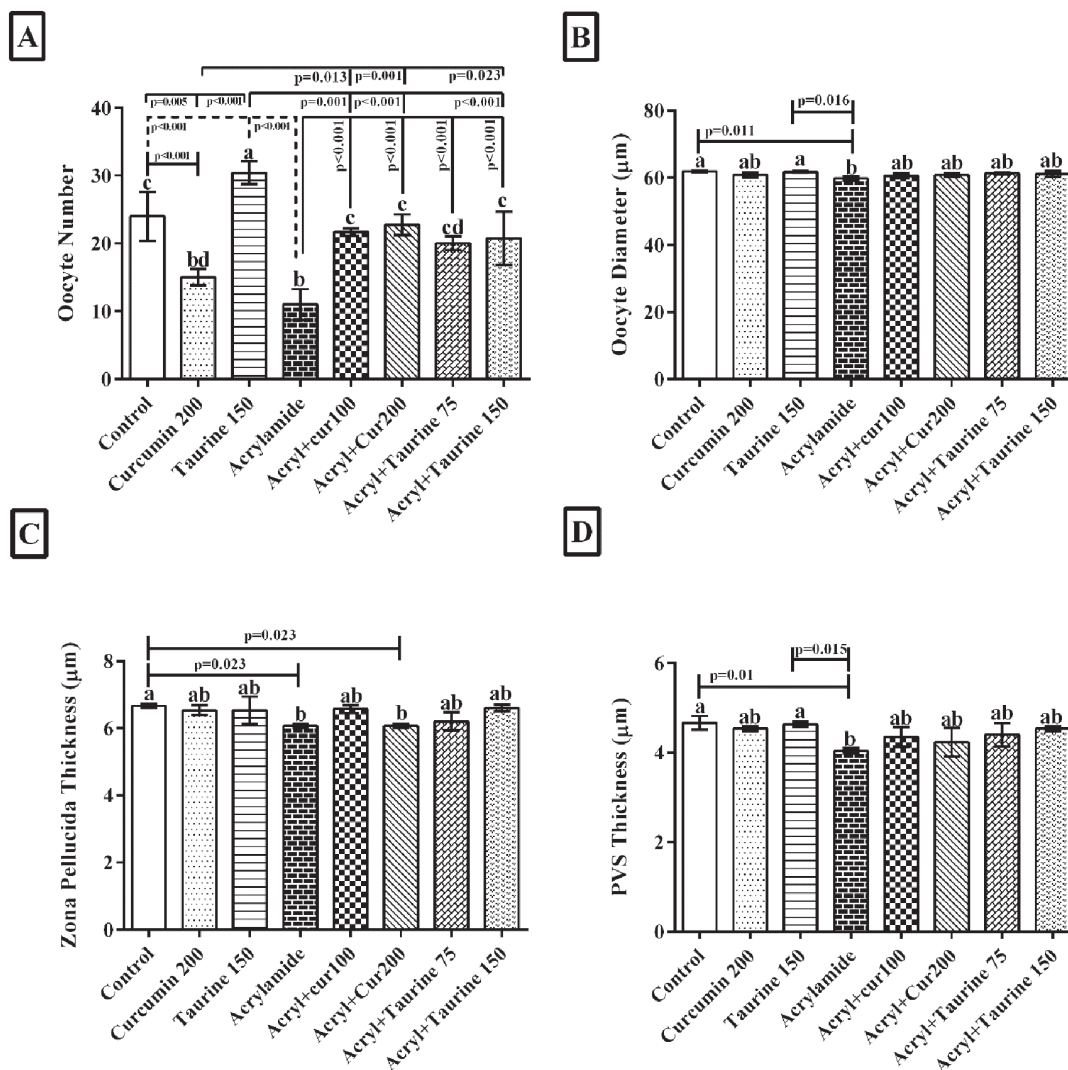
#### Oocyte Assessment

According to figure 1, the mean number of oocytes, oocyte diameter, zona pellucida, and perivitelline space (PVS) in the group that received acrylamide showed a significant decrease compared to the control group ( $P < 0.001$ ). A significant increase in the mean number of oocytes was observed in the taurine group compared to the control group ( $P < 0.001$ ). Additionally, a significant increase in the mean number of oocytes was observed

**Table 1:** Primer sequences for the forward and reverse genes involved in the autophagy process

Gene	Sequence	Size (bp)	Annealing Temperature
<i>M-Atg5</i>	Forward: AACTGAAAGAGAAGCAGAACCA	105	60
<i>M-Atg5</i>	Reverse: TGTCTCATAACCTTCTGAAAGTGC		
<i>M-beclin 1</i>	Forward: AATCTAAGGAGTTGCCGTTATAC	187	60
<i>M-beclin 1</i>	Reverse: CCAGTGTCTTCAATCTTGCC		
<i>M-B-actin</i>	Forward: AGTGTGACGTTGACATCCGT	120	60
<i>M-B-actin</i>	Reverse: TGCTAGGAGCCAGAGCAGTA		
<i>Atg8</i>	Forward: GACCATCCAAGGACTCATTGAC	131	60
<i>Atg8</i>	Reverse: GGGCAAAGGACTGATTACATA		
<i>LC3</i>	Forward: CCACCAAGATCCCAGTGATTATAG	125	60
<i>LC3</i>	Reverse: CAAGCGCCGTCTGATTATCT		

M: Mouse; bp: Base pair; Atg: Autophagy-related gene; LC3: Light chain 3



**Figure 1:** The number, diameter, thickness of the zona pellucida, and PVS of the oocyte in the studied groups. Data are presented as mean±SD, n=12. <sup>a,b,c</sup>There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (P<0.05).

in the group receiving acrylamide+curcumin 100 and 200 mg/Kg and the group that received acrylamide+taurine 75 and 150 mg/Kg compared to the group that received acrylamide alone (P<0.001). There was no significant difference in the mean number of oocytes between the groups that received acrylamide+taurine 150 mg/Kg and those receiving acrylamide+curcumin 100 and 200 mg/Kg. The oocyte diameter was significantly increased in taurine 150 mg/Kg compared to acrylamide (P<0.001). Furthermore, in the acrylamide groups treated with curcumin 100 and 200 mg/Kg and taurine 75 and 150 mg/Kg, no significant difference was observed in the mean of oocyte diameter.

The mean thickness of the zona pellucida in the groups that received acrylamide and acrylamide+curcumin 200 mg/Kg showed a significant decrease compared to the control group (P=0.023).

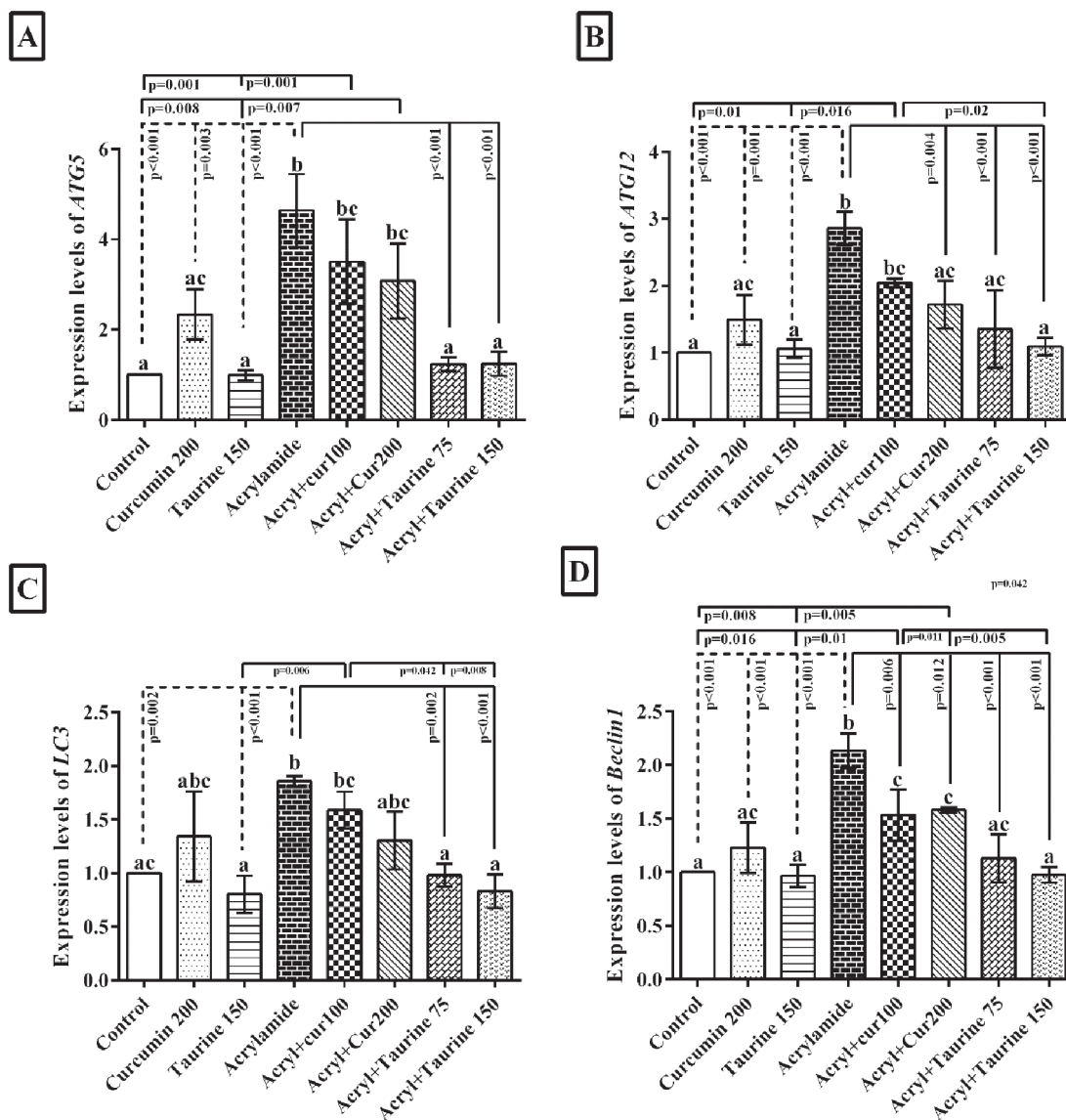
The PVS thickness was significantly decreased in the acrylamide groups in comparison to the control group (P<0.001).

#### Autophagy Gene Expression Analysis

According to figure 2A, a significant upregulation in the expression levels of the *ATG5* gene was observed in the oocytes of the acrylamide-treated group compared to other groups. The acrylamide+curcumin 100 and 200 mg/Kg groups also showed a significant increase in the expression level of *ATG5* genes compared to control groups (P=0.001 and P=0.008). The groups receiving acrylamide+curcumin 100 and 200 mg/Kg showed a significant increase in *ATG5* gene expression compared with taurine group (P=0.001 and P=0.007, respectively). There was no significant difference in the expression level of *ATG5* gene in oocytes of the control groups and taurine and curcumin groups (P>0.999 and P=0.149, respectively).

The expression level of the *ATG12* gene also





**Figure 2:** The related mRNA expression of *ATG5*, *ATG12*, *LC3*, and *Beclin1* genes in oocytes in different groups including (control, curcumin 200 mg/Kg, taurine 150 mg/Kg, acrylamide 50 mg/Kg, acrylamide 50 mg/Kg+curcumin 100 mg/Kg, acrylamide 50 mg/Kg+curcumin 200, acrylamide 50 mg/Kg+taurine 75 mg/Kg, acrylamide 50 mg/Kg+taurine 150). Data are presented as mean±SD, n=12. <sup>a,b,c</sup>There were no significant differences between the columns containing at least one similar letter. However, different letters reveal a significant difference (P<0.05).

showed a significant increase in the acrylamide-treated group compared to other groups (P=0.001), but the acrylamide+curcumin 100 mg/Kg group was not significant with acrylamide group (P=0.057). A significant increase in *ATG12* gene expression was observed in oocytes from the acrylamide+curcumin 100 mg/Kg groups compared with the acrylamide+taurine 150 mg/Kg group (P=0.021). However, this difference was not significant in the other treatment groups. The acrylamide+curcumin 100 mg/Kg group (P=0.01) showed a substantial increase in *ATG12* gene expression compared to the control group. No significant differences were observed in *ATG12* gene expression levels in oocytes from the control group and the taurine and curcumin

groups (P>0.999 and P=0.484) (figure 2B).

A significant increase in *LC3* gene expression in oocytes was observed in the treatment group receiving acrylamide+curcumin at 100 mg/Kg compared to the group receiving acrylamide+taurine at 150 mg/Kg and 75 mg/Kg at the level of (P=0.008 and P=0.042, respectively). The group receiving acrylamide+curcumin at 100 mg/Kg (P=0.051) showed a significant increase in *LC3* gene expression compared to the control group. Additionally, the group receiving acrylamide+curcumin at 100 mg/Kg (P=0.006) showed a significant increase in *LC3* gene expression compared to the healthy group receiving taurine. No significant difference in

*LC3* gene expression in oocytes was observed between the control group and the healthy group receiving taurine and curcumin ( $P=0.933$  and  $P=0.510$ , respectively).

The expression level of the *Beclin1* gene showed a significant increase in the oocytes of the acrylamide-treated group compared to control, curcumin, taurine, acrylamide+taurine 75, 150 mg/Kg groups ( $P=0.001$ ) (figure 2C). The acrylamide+curcumin 100 and 200 mg/Kg groups also showed a significant increase compared to the acrylamide group ( $P=0.006$  and  $P=0.012$ , respectively).

A significant decrease in *Beclin1* gene expression was shown in acrylamide+taurine 150 mg/Kg groups compared with the acrylamide+curcumin 100 mg/Kg ( $P=0.011$ ) group and the acrylamide+curcumin 200 mg/Kg ( $P=0.005$ ) group. The acrylamide+curcumin 100 mg/Kg ( $P=0.016$ ) group and the acrylamide+curcumin 200 mg/Kg ( $P=0.008$ ) group showed a significant increase in *Beclin1* gene expression compared with the control group. Additionally, the acrylamide+curcumin (100 mg/Kg) group ( $P=0.01$ ) and the acrylamide+curcumin 200 mg/Kg ( $P=0.005$ ) group showed a significant increase in *Beclin1* gene expression compared with the taurine group. There was no significant difference in the expression level of the *Beclin1* gene in oocytes between the control and the taurine and curcumin groups ( $P>0.999$  and  $P=0.662$ ) (figure 2D).

The results indicated a significant reduction in the expression levels of *LC3*, *ATG12*, *ATG5*, and *Beclin1* genes in the oocytes of the group that received acrylamide+taurine 75 and 150 mg/Kg compared to the group that received acrylamide alone ( $P<0.001$ ). The results also showed a significant decrease in the expression levels of *Beclin1* ( $P=0.011$  and  $P=0.05$ ) and *ATG5* ( $P=0.004$  and  $P=0.021$ ) genes in the group that received acrylamide+taurine 150 mg/Kg compared to the group receiving acrylamide+curcumin 100 and 200 mg/Kg. Additionally, a significant reduction was observed in the expression levels of *LC3* and *ATG12* genes in the group that received acrylamide+taurine 150 mg/Kg compared to the group that received acrylamide+curcumin 100 mg/Kg ( $P=0.008$  and  $P=0.20$ , respectively).

## Discussion

The results of this investigation demonstrated that, in comparison to the control group, acrylamide significantly decreased the number of oocytes, oocyte diameter, zona pellucida thickness, PVS, and significantly increased the

expression of the genes *LC3*, *ATG12*, *ATG5*, and *Beclin1*. When taurine was administered instead of curcumin, the mice who received acrylamide fared better. The taurine group had a significantly larger average number of oocytes than the control group. A significant increase in the average number of oocytes was observed in the taurine group compared to the curcumin group. Numerous intracellular processes have been identified in the biological system to adapt to different types of cell stress including the production of some genes and proteins linked to antioxidant enzymes at altered levels, elevated heat shock protein levels, metallothionein synthesis, apoptosis, and autophagy.<sup>24, 25</sup>

Song and colleagues discovered that acrylamide reduces the amounts of glycolytic intermediates, which in turn reduces glycolysis/gluconeogenesis, raises ROS content, increases apoptosis, and inhibits autophagy. They identified the fundamental impacts of ACR on autophagy in their investigation. ACR suppresses autophagy by obstructing autophagic flow, as suggested by the significant increase of *LC3-II* and *p62* levels. According to the results of metabolomics, exposure to ACR decreased the pace at which glycolysis and the TCA cycle were metabolized, while, on the other hand, increasing the metabolism of fatty acids and amino acids. In addition, ACR promoted inflammation, cell death, and ROS generation.<sup>26</sup>

Natural products such as curcumin and taurine can be used in an autophagy-dependent manner to increase the rate of embryo implantation. Only a small number of research have, however, examined autophagy as a key mechanism behind the enhancement of embryo implantation and, consequently, the use of natural products in the treatment of female infertility.<sup>27</sup> The novelty of this study is using a combination of these natural products and evaluation of autophagy-related genes in oocytes.

A study showed that adding taurine to the culture medium enhances the rate of fertilization and blastocyst development when compared to the control group.<sup>28</sup> These outcomes are in agreement with our investigation. According to this study, a significant increase in the mean number of oocytes was observed in the taurine group compared to the control group. Together, curcumin's numerous biological activities, low toxicity, and reduced side effects over synthetic drugs make it a potentially helpful treatment for female reproductive disorders, contraception, and chemotherapy or chemo-preventive treatment, and safeguards against lowering semen quality parameters.<sup>29</sup> In normal cells, curcumin mainly induces autophagy through

different mechanisms to promote cell survival. Meanwhile, in cancer cells, curcumin causes excessive autophagy stimulation,<sup>30</sup> leading to cell death.<sup>31</sup> In the present study, curcumin, especially at high doses, caused a decrease in the number of oocytes, and to some extent, the expression of autophagy pathway genes. Therefore, it can be stated that curcumin in high doses leads the cell toward the apoptotic pathway, which requires further studies in this regard. Curcumin therapy at doses of 100 and 200 mg/Kg effectively restores antioxidant gene expression, with a substantial improvement at the 200 mg/Kg dose over the 100 mg/Kg dose. Several studies have demonstrated the efficiency of curcumin in alleviating oxidative stress-related problems, including parameters such as total antioxidant capacity, malondialdehyde, and superoxide dismutase under physiological settings.<sup>10, 32</sup>

Gao and colleagues found that taurine can prevent autophagy by activating the mammalian target of rapamycin (*mTOR*)-peroxisome proliferator-activated receptor- $\gamma$  signaling pathway. When calcium oxalate crystals damage the epithelial cells of renal tubules, taurine can activate the *Akt/mTOR* (protein kinase B known as *Akt*) signaling pathway to block ROS-dependent autophagy.<sup>33</sup> Taurine also reduces the production of *LC3-II* protein and green fluorescent protein -*LC3* in porcine kidney 15 cells, which decreases the autophagy effects caused by Hercotoxin A.<sup>34</sup> In the present study, significant reductions in *LC3* expression were observed in the group treated with 75 mg/Kg and 150 mg/Kg taurine, indicating a decrease in the number of autophagosomes. This finding is consistent with previous studies in that taurine can inhibit autophagy.<sup>35, 36</sup> Thus, compared to the acrylamide group, the simultaneous treatment of taurine and acrylamide resulted in a significant reduction in the number of autophagosomes and a considerable decrease in the expression of autophagy-related genes *Atg5*, *Atg12*, *LC3*, and *Beclin-1*, suggesting that it can reduce the autophagic blockade caused by acrylamide.

According to a 2020 study by Aldawood and others., there was a considerable increase in the mRNA levels of the *ATG5* and *ATG12* genes in ovarian tissue following high-dose acrylamide injection.<sup>3</sup> These findings align with Tan and colleagues' investigation.<sup>37</sup> However, despite autophagosomes in acrylamide-treated animals as shown by electron microscopy sections, acrylamide treatment had no effect on *LC3*. The function of the autophagic mechanism in defending the oocytes was also covered in this investigation.<sup>38</sup> Despite this, our results showed

that *LC3* levels increased when acrylamide was administered. This is significant since *LC3* is an autophagy marker protein mainly involved in the maturation, formation, and destruction of autophagosomes.<sup>28</sup> Taken together, these results demonstrate that autophagy affects the oocyte by inducing apoptosis in the corpus luteum or follicular atresia analysis process. It has been established that gonadotropin is required for both processes to occur in the ovaries of mice and that autophagic activation is facilitated by the estrogen receptors (ER1 and ER2).<sup>28</sup> Based on these findings, we propose that the autophagy process works with granulosa cell death and apoptosis caused by acrylamide. According to Yang and colleagues, these substances can decrease anti-autophagic proteins including *Akt* and *mTOR4* in ovarian cells while simultaneously inducing autophagy repair and activating the adenosine monophosphate-activated protein kinase pathway.<sup>39</sup> On the other hand, autophagy can be induced by high doses of acrylamide treatment, as evidenced by an increase in the mRNA levels of autophagy-related genes; however, autophagy inhibition may eventually result in cell death.<sup>40</sup> This autophagy blockage can happen by inhibiting either autophagosome-lysosome fusion or lysosomal degradation, which calls for more research in this field.

Our study was limited in that protein expression by western blot was not assessed due to insufficient budget. Furthermore, the development of fertilized oocytes to the blastocyst stage should be evaluated in conjunction with the expression of *ATG* genes at each stage in the future. However, further studies are needed in this area.

## Conclusion

The results indicated that acrylamide negatively impacts oocyte viability and that autophagy-related genes are expressed at higher levels. Taurine may stimulate autophagosomes to fuse with lysosomes by removing autophagic obstruction, thereby potentially accelerating the autophagy process and protecting against oxidative stress. Taurine is more effective than curcumin at reducing the harmful effects of acrylamide. As a result, taurine can be regarded as a potential therapeutic drug for acrylamide-induced infertility.

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### Authors' Contribution

M.DZ: Data collection and interpretation, drafting and reviewing the manuscript; Z.KH: Study design, data analysis, drafting and reviewing the manuscript; MA.E: Data analysis and interpretation, drafting and reviewing the manuscript; All authors contributed to the study's conception and design and commented on the last versions of the manuscript and read and approved the final manuscript and provided an agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest:** None declared.

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