RESEARCH ARTICLE

Glycine-L-Proline Media Supplementation Reduces Reactive Oxygen Species and Improves Preimplantation Development in Mouse Embryos

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Abstract In vitro embryo culture systems often expose embryos to elevated oxidative stress levels, compromising development. Glycine (Gly) and L-Proline (L-Pro), naturally occurring amino acids with antioxidant properties, have been shown to mitigate reactive oxygen species (ROS). This study aimed to assess the individual and combined effects of Gly and L-Pro supplementation on preimplantation development in mouse embryos. Two-cell stage BALB/C mouse embryos were cultured in M16 medium supplemented with either Gly or L-Pro at concentrations of 0.2, 0.4, and 0.6 mM, and a combined group of 0.6 mM Gly + 0.6 mM L-Pro. Developmental progress to the 8-cell, morula, and blastocyst stages was recorded. Mitochondrial activity and ROS levels were quantified using MitoTracker Red and H2DCFDA assays, respectively. The combined treatment group exhibited significantly higher rates of normal morphology (94.3% 8-cell, 85.7% morula, 72.9% blastocyst) and developmental competence (92.9% blastocyst formation, 55.7% hatching) compared to controls ($p < 0.01$). ROS levels were lowest in the combined group (22.24 ± 1.54 pixel x10^5) and mitochondrial intensities were highest (105.1 ± 5.83 pixel x10^5), indicating improved mitochondrial activity and reduced oxidative stress. These findings suggest a complementary interaction between Gly and L-Pro in enhancing embryo quality and development by modulating oxidative stress and mitochondrial function. This novel supplementation strategy could inform future improvements in assisted reproductive technologies.

Keywords: Assisted Reproductive Technology, Embryo Development, Glycine, L-Proline, Mitochondria, Reactive Oxygen Species.

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Introduction

Infertility remains a growing global health issue, with increasing numbers of couples seeking assistance through Assisted Reproductive Technology (ART) to conceive [1]. A key factor influencing ART success is the quality of embryo culture media, which directly affects embryonic viability and developmental outcomes. Optimal media conditions are essential for supporting critical embryonic features such as blastomere symmetry, low fragmentation, and appropriate zona pellucida thickness [2]. The developmental potential of an embryo can also be predicted through morphometric analysis of blastocysts, particularly evaluating the inner cell mass (ICM) and trophectoderm (TE) cell counts [3].

Numerous studies have emphasized the nutritional environment during embryo culture as a determinant of implantation success and pregnancy rates [4]. Amino acids in the female reproductive tract, including glycine and L-proline, play a vital role in supporting preimplantation development and implantation [5]. Glycine (Gly) is highly abundant in mammalian oviducts [6] and has been found to enhance embryo survival, promote blastocyst development, and support mitochondrial stability [19]. Likewise, L-proline (L-Pro) has demonstrated antioxidant capacity by scavenging Reactive Oxygen Species (ROS) and modulating mitochondrial metabolism [17]; [23].

ROS are byproducts of mitochondrial respiration and are unavoidably elevated during in vitro culture [12]. Excessive ROS can lead to cellular damage, compromised DNA integrity, and embryonic arrest [13]; [14]. Maintaining redox balance through antioxidant support is essential for preserving embryonic viability and function [15]. Non-essential amino acids such as Gly and L-Pro serve dual roles in protein biosynthesis and cellular antioxidant defence [9].

Furthermore, mitochondria are crucial for ATP production and embryonic metabolic regulation. Dysfunctional mitochondria impair glycolysis, amino acid metabolism, and energy synthesis, leading to suboptimal embryo development [21]. Both Gly and L-Pro have been linked to enhanced mitochondrial performance in vitro [22]; [23]. Prior studies have explored combinations of amino acids, such as glutathione with cysteine, to reduce oxidative stress and improve blastocyst outcomes [24].

However, limited data exist on the complementary effects of Gly and L-Pro in combination, particularly regarding their optimal concentrations for specific strains such as BALB/C mice. Therefore, this study investigates the individual and combined roles of Gly and L-Pro at defined concentrations in improving preimplantation development, reducing oxidative stress, and enhancing mitochondrial activity in mouse embryos cultured in vitro.

Materials and Methods

In this study, 48 female mice (6 mice/group) of the BALB/C strain at 6-12 weeks of age, weighing 25-30 grams, were used as embryo donors, while male mice of the same strain at 8-12 weeks of age were used for natural mating. The experiments were performed under ethical approval from the University Committee on Animal Research and Ethics (CARE), Universiti Teknologi Mara, Puncak Alam Campus (UiTM CARE 5/2020/ (324/2020).

The temperature in the mouse holding room was maintained at 27 ^{0}C with a 12:12 light: dark cycle. They were provided with water and standard rodent maintenance diet (Altromin Spezialfutter GmbH and Co., Lage, Germany) *ad libitum* daily. The experimental overview is shown in Figure 1. Two-cell embryos were selected and divided into 7 groups for in vitro culture and observation of preimplantation development until the hatched blastocyst stage. For this study, each group of Gly (Sigma, USA) and L-Pro (Sigma, USA) was divided into three concentrations (0.2 mM, 0.4 mM, and 0.6 mM), considered as treated groups [1]. A group without any treatment acts as the control group.

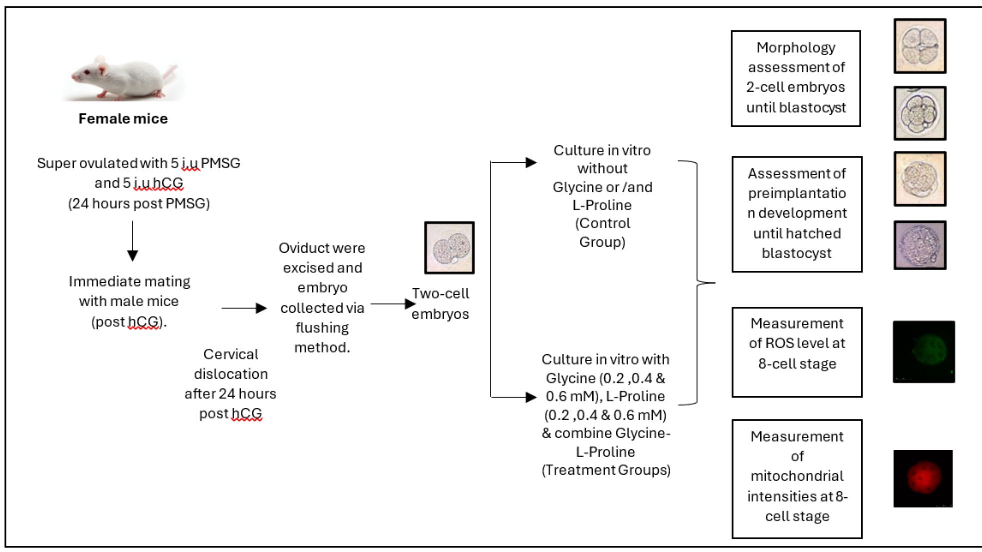

Figure 1. Diagram of Experimental Design

Superovulation and Embryo Collection

Female mice were superovulated by intraperitoneal injection (IP) of 5 IU PMSG (Prospec, HOR:272) followed by 5 IU hCG (Prospec, HOR;250) after 48 hours. Each female mouse was mated with fertile male mice immediately after hCG injection. The presence of a vaginal plug at 24 hours indicates that fertilization has occurred, and female mice were euthanized by cervical dislocation at 48 hours post-hCG. All donors were managed under uniform collection conditions using the oviduct flush method to collect 2-cell embryos. The embryos obtained from multiple donors were pooled before allocation to experimental groups to ensure adequate sample size per treatment. To reduce donor-related bias, a total of 515 two-cell embryos from all mice with normal morphology were randomly distributed among groups for the study.

Morphological Assessment and Grading of Preimplantation Embryos

The morphology of the embryo at the 2-, 4-, 8-, morula, and blastocyst stages was recorded under an inverted microscope (Leica DM IRB, BZ:05) connected to the PC software (PixeLINK). The embryos were graded as normal or abnormal for all embryonic stages, and the grading of embryos at each stage was assessed according to the ASEBIR morphological grading system [2].

Preimplantation Development Assessment

The two-cell embryos were cultured *in vitro* in an M16 medium with and without the addition of Gly and L-Pro amino acids. The culture media were coated with mineral oil (Sigma, USA) and maintained for five days in an atmosphere of 5% CO_2 at 37 ^{0}C in the incubator. The embryo development rate of embryos was calculated as a percentage (%) of developed embryos based on the total number of developed embryos.

Measurement of Mitochondrial Distribution and Intensities

The best embryonic development results of the individual Gly and single L-Pro groups and the combined group were further analyzed to measure the distribution and intensity of mitochondria using MitoTracker™ Red CMXRos (Cat. M7512, Invitrogen, MA, USA). It is a sensitive indicator of relative changes in mitochondrial membrane potential, indicating the presence of active mitochondria. A total of 30 eight-cell stage embryos from each group were used. The embryos were fixed in 4% formaldehyde and incubated for 24 h. They were then permeabilized with 1% Triton-X in PBS, followed by labeling with MitoTracker Red. They were washed twice with PBS, mounted on slides with ProLong Gold (Invitrogen, USA), and stored overnight at 4ºC. The slides were analyzed with the Confocal Laser Scanning Microscope (CLSM) (Leica TCS SP5 AOBS, Germany) with excitation at 543 nm and emission at 560 nm. The intensities of the image were quantified with ImageJ by the mean grey values of the fluorescence. Background fluorescence values were deducted from the final measurements before conducting statistical comparisons between groups.

Measurement of Intracellular ROS

The best embryonic development results for each Gly and L-Pro group were analyzed using ROS content, together with the control and combined groups. A total of 30 eight-cell stage embryos from each group were used to measure intracellular ROS using the 2'7' dichlorofluorescein diacetate (H2DCFDA) fluorescence assay. The embryos were preserved overnight for at least 4 hours in 4% paraformaldehyde at 4 °C. They were then rinsed twice with PBS-1% BSA and incubated with H2DCFDA fluorescence probe (Cat. C-400, Life Technologies, Thermo Fisher Scientific, MA, USA) for 30 min at 37 ^{0}C and counterstained with 6-diamidino-2-phenylindole (DAPI) to stain the nucleus. Embryos were rinsed twice or more with PBS-1% BSA and fixed with an antifading medium (Prolong Gold Antifading Agent; Molecular Probes, Life Technologies, CA, USA). Fluorescence images were captured using a Confocal Laser Scanning Microscope (Leica CLSM) with a UV filter at 460 nm to measure the fluorescence signal. The intensities of the image were quantified with ImageJ using the mean grey values of the fluorescence. Background fluorescence values were deducted from the final measurements before conducting statistical comparisons between groups.

Statistical Analysis

Statistical analyses were performed with SPSS (Statistical Package for Social Science) Version 26.0 and GraphPad Prism 8 for Windows, version 8.0.1 (GraphPad Software., LA, USA) was used for graphical representations. All stages of embryos were assessed qualitatively. Statistical difference in embryo development was determined using a Chi-square test, with significance at $p<0.05$. Fluorescence intensity in both ROS and mitochondrial analysis were determined using one-way ANOVA followed by Tukey's multiple comparison test. Data were expressed as means ± standard error for the mean (SEM). Results were considered statistically significant at $p<0.05$.

Results and Discussion

Embryo quality and developmental competence are the primary indicators for selecting the best culture media. Additionally, mitochondrial activity and ROS levels were analysed to predict the optimal amino acid concentration for embryo culture.

Effect of Different Concentrations of Glycine and L-Proline as Single and Combined Preparations on the Morphology of Embryos

The morphology of the embryos was categorised as normal or abnormal for all embryonic stages according to the embryo classification system [2]. Uniform size and number of blastomeres with a certain degree of fragmentation were the most important aspects used in the classification. A uniform size and an even number of blastomeres, with less than 10% fragmentation, were considered normal. Abnormal morphology is defined by an odd number and size of blastomeres with more than 10% fragmentation. Normal 2-cell embryos were selected to assess their developmental potential through the blastocyst stage. Table 1 and Figure 2 illustrate the normal and abnormal characteristics of the 2-, 4-, and 8-morula and blastocyst stages.

Table 1 shows that the percentage of normal developmental stages was higher in all supplementation groups than in the control group. The percentage of normal 4-cell embryos increased at all concentrations from 0.2 mM, 0.4 mM, and 0.6 mM of single Gly and L-Pro groups. At 0.6 mM of the individual Gly and L-Pro groups, the percentage of normal blastocysts was highest (69.8% and 59.6%, respectively). Remarkably, combined Gly-L-Pro supplementation resulted in the highest percentage of normal 8-cell, morula, and blastocyst formation among all groups (94.3%, 85.7%, and 72.9%, respectively).

Table 1. Morphology Assessment of Preimplantation Embryos

Group	4-cell Total number (%)		8-cell Total number (%)		Morula Total number (%)		Blastocyst Total number (%)	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Control	54 (78.3%)	15 (21.7%)	49 (71.0%)	20 (29.0%)	40 (57.9%)	29 (42.1%)	28 (40.6%)	41 (59.4%)
0.2 mM L-Proline	53 (80.3%)	13 (19.7%)	47 (83.9%)	9 (16.1%)	37 (71.2%)	15 (28.8%)	22 (55.0%)	18 (45.0%)
0.4 mM L-Proline	54 (81.8%)	12 (18.2%)	49 (79.0%)	13 (21.0%)	41 (71.9%)	16 (28.1%)	27 (58.7%)	19 (41.3%)
0.6 mM L-Proline	55 (84.6%)	10 (15.4%)	50 (84.7%)	9 (15.3%)	42 (73.7%)	15 (26.3%)	28 (59.6%)	19 (40.4%)
0.2 mM Glycine	51 (86.4%)	8 (13.6%)	45 (78.9%)	12 (21.1%)	43 (75.4%)	14 (24.6%)	31 (63.3%)	18 (36.7%)
0.4 mM Glycine	55 (91.7%)	5 (8.3%)	50 (86.2%)	8 (13.8%)	45 (80.4%)	11 (19.6%)	35 (68.6%)	16 (31.4%)
0.6 mM Glycine	56 (93.3%)	4 (6.7%)	52 (83.9%)	10 (16.15)	48 (80.0%)	12 (20.0%)	37 (69.8%)	16 (30.2%)
Combined 0.6 mM Glycine + 0.6 mM L-Proline	68 (97.1%)	2 (2.9%)	66 (94.3%)	4 (5.7%)	60 (85.7%)	10 (14.3%)	51 (72.9%)	19 (27.1%)
Images of the embryo								
Morphology	Embryo with 4 even sized blastomeres and presence of ≤10% fragmentation	Embryo with 4 uneven-sized blastomeres and presence of >10% fragmentation	Embryo with 8 even sized blastomeres and presence of ≤10% fragmentation	Embryo with 8 uneven-sized blastomeres and presence of >10% fragmentation	Morula showing full compaction. No blastomere can be distinguished	Morula showing partial compaction. Blastomeres can be distinguished	TE cells are homogenous, cohesive, and numerous cells. Bigger blastocoel cavity	TE cells are less homogenous and fewer. Smaller blastocoel cavity

Embryo development and survival depend on the nutrients in the embryo culture media, such as minerals, glucose, lactate, antibiotics, and proteins, including amino acids [3], [4]. Amino acids are very abundant in the female reproductive tract, where they are produced by epithelial cells [5] in the oviduct and uterus. Since different mammalian species have different physiological components due to the requirements of embryo growth, the concentration of amino acids also varies between the species [6]. Men *et al*., (2023)reported that the presence of 1mM amino acid has a positive effect on the development of rat embryos but not on mouse embryos. The explanation for this is that mouse embryos developing *in vitro* do not require enriched amino acids, as in the case of rat and bovine embryos [7].

Therefore, in this study, different concentrations of L-Pro and Gly, ranging from 0.2 mM to 0.6 mM, were tested in BALB/c *in vitro* embryo culture. The current study showed that the addition of 0.6 mM Gly to M16 media individually improved the normal morphology of the embryos compared to the single treatment of L-Pro and the control group. In addition, the combination of 0.6 mM L-Pro and 0.6 mM Gly increased the percentage of normal morphology, thereby promoting blastomere symmetry and reducing fragmentation. Good-quality embryos potentially lead to high pregnancy rates [8] and more live births [9].

On the other hand, fragmentation refers to the enucleated structures of the cytoplasm formed during cell division that lack DNA [10] in embryo morphology. Several factors, such as apoptotic cell death, [11] presence of ROS [12] the disruption of the cytoskeleton [13], the formation of vesicles and micronuclei [14] and the uneven distribution of mitochondria [15] lead to fragmented embryos. The consequences of fragmented embryos are increased chromosomal abnormalities and reduced normal blastocyst formation, which can ultimately lead to unsuccessful implantation and pregnancy failure [16].

Effect of Different Concentrations of Glycine and L-Proline as Single and Combined Preparations on Preimplantation Development

The effects of single and combined supplementation of Gly and L-Pro on embryonic development are shown in Table 2. Although the concentration range of single L-Pro showed no statistical differences between groups, there were significant differences in developmental competence at the 8-cell, morula, and blastocyst stages compared with the control group ($p < 0.05$). The same results were also observed in the individual single Gly groups compared with the control group for preimplantation development at the 8-cell, morula, and blastocyst stages ($p < 0.01$). The results were found highly significant in the groups with 0.2 mM, 0.4 mM and 0.6 mM of single Gly as well as combined Gly-L-Pro group compared to the control group in morula (96.6%, 93.3%, 98.3% and 100% respectively) and blastocyst (86.4%, 85.0%, 88.3% and 92.9% respectively) stages ($p<0.01$).

The combined Gly-L-Pro group showed a significantly greater difference than the individual L-Pro and single Gly groups, as well as the control group ($p<0.01$). The highest development rate was observed in the combined Gly-L-Pro group, which produced 100% morulae and 92.9% blastocysts.

Both 0.6 Mm of Gly and L-Proline individually can increase developmental competence. However, the results of this study show that combined supplementation with Gly-L-Pro can further improve preimplantation development, including 8-cell, morula, and blastocyst formation. In other animal studies, it was reported that the addition of 6.0 mM Gly could support greater blastocyst formation in porcine embryos [17] while 1.0 mM Gly was found to have improved the development of early and late-stage bovine embryos [18]. Even though the 8-cell, morula, and blastocyst formation in the L-Pro group is not comparable to the Gly group, the intake of amino acids such as L-Pro improves the quality and development of embryos compared to the non-supplemented group.

Previous studies have found that Gly and L-Pro play an important role in cell proliferation and differentiation by activating mTOR1 (mammalian target of rapamycin [19], [20]. Gly [21] and L-Pro [22] amino acid sensors (GLYT1 and SLC6a19, respectively) allow the influx of Gly and L-Pro and activate the Arf1 regulator, enabling Rheb-GTP to activate mTORC1 [20], [23]. Activated mTORC1 promotes protein and lipid synthesis in the embryonic cell, which eventually promotes preimplantation development. The presence of both amino acids in the combined group activates both GLYT and SLC sensors, thereby activating the MTOR pathway.

These could be the reasons for the improved preimplantation development in the combined treatment compared to the control and single treatments. The percentage of compacted embryos and blastocysts, as well as the number of inner cell mass (ICM) cells, also improved in the combined group. This suggests that the presence of combined Gly-L-Pro may have promoted cell growth and reduced the number of arrested embryos.

Table 2. Developmental Competence of Preimplantation Embryos

Group	Preimplantation Development Stage: n (%)				
	2-cell	8-cell	Morula	Blastocyst	Hatched Blastocyst
Control	69 (100.0%) [a]	50 (72.5%) [a]	41 (59.4%) [a]	31 (44.9%) [a]	8 (11.6%) [a]
L-Proline 0.2 mM	66 (100.0%) [a]	57 (84.8%) [b]	55 (83.3%) [b]	40 (60.6%) [a]	15 (22.7%) [a]
L-Proline 0.4 mM	66 (100.0%) [a]	61 (92.4%) [b]	57 (86.4%) [b]	46 (69.7%) [b]	18 (27.3%) [b]
*L-Proline 0.6 mM	65 (100.0%) [a]	59 (90.8%) [b]	55 (84.6%) [b]	47 (72.3%) [b]	20 (30.8%) [b]
Glycine 0.2 mM	59 (100.0%) [a]	57 (96.6%) [c]	57 (96.6%) [c]	51 (86.4%) [c]	22 (37.3%) [b]
Glycine 0.4 mM	60 (100.0%) [a]	58 (96.7%) [c]	56 (93.3%) [c]	51 (85.0%) [c]	23 (38.3%) [b]
*Glycine 0.6 mM	60 (100.0%) [a]	59 (98.3%) [c]	59 (98.3%) [c]	53 (88.3%) [c]	25 (41.6%) [b]
Glycine-L-Proline	70 (100.0%) [a]	70 (100.0) [c]	70 (100%) [c]	65 (92.9%) [c]	39 (55.71%) [c]

(* L-Proline and Glycine concentration that was selected as the combined group.)
Different superscript letters within a column indicate statistically significant differences (a-b; $p< 0.05$ and a-c; $p < 0.01$)

Effect of Different Concentrations of Glycine and L-Proline as Single and Combined Preparations on the Hatching Rate of Blastocysts

All groups supplemented with amino acids produced a higher hatching rate compared to non-supplemented embryo culture. In the 0.4 mM and 0.6 mM L-Pro and all single Gly groups, the hatching rate was significantly higher than in the control group ($p < 0.05$). In agreement with the morphological assessment of embryos, the combined Gly-L-Pro group had a higher hatching rate of blastocysts than the control group (55.71% vs 11.6%; $p<0.01$). This faster development of blastocysts is triggered by the presence and number of active mitochondria [24].

The hatching of blastocysts is one of the most important events that determines a successful pregnancy. An enlargement of the blastocyst by 2-3 times its original size, due to fluid absorption and an increase in cell number, is the main factor leading to hatch blastocyst. The trophoblast cells develop opposite the ICM pole and break through the zona pellucida. The specialized TE cells that break through the zona pellucida have special characteristics, such as plump cells at the hatching point and myriads of microvilli on the surface that interact with the zona pellucida [25]. Such a mechanism is crucial for the implantation process. In this study, the highest percentage of hatched blastocysts observed with Gly-L-Pro supplementation may be due to the complementary effects of the two amino acids on cell proliferation, thereby contributing to blastocyst expansion. Our results are consistent with another study reporting an improved blastocyst hatching rate in porcine embryos in the presence of Gly [26].

Evaluation of Mitochondrial Distribution and Intensity of 8-Cell Embryos in 0.6 Mm Glycine and L-Proline as Single and Combined Preparations

For this study, the analysis of the fluorescence intensities for mitochondria in 8-cell embryos is shown in Figures 2 and 3 (A-D). It was found that the mitochondrial intensities in both the single Gly and L-Pro groups (90.63 ± 4.048 and 84.04 ± 4.346, respectively) were significantly higher than those in the control group ($p < 0.01$). The combined Gly-L-Pro group showed the highest fluorescence intensity (105.1 ± 5.827 pixel $x10^5$) and was significantly higher than the control group ($p<0.01$). This indicates that the Gly-L-Pro supplementation in embryo culture medium increased mitochondrial activity.

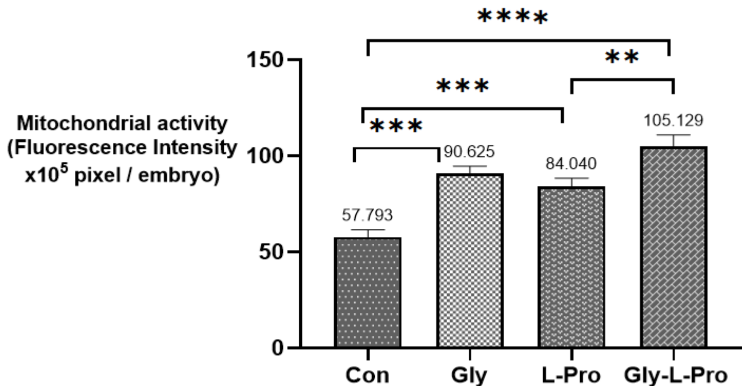

Figure 2. Mitochondrial intensities of the 8-cell embryos as of con (control), Gly (0.6 mM Gly), L-Pro (0.6 mM L-Pro), and combined Gly-L-Pro (0.6 mM Gly + 0.6mM L-Pro) presented in fluorescence intensity (in pixels x 10^5). Data were presented as mean ± SEM to reflect the precision of the estimated group mean across biological replicates. Individual values were illustrated. **, *** and **** indicate significant difference respectively at $p < 0.01$, $p < 0.001$ and < 0.0001 levels.

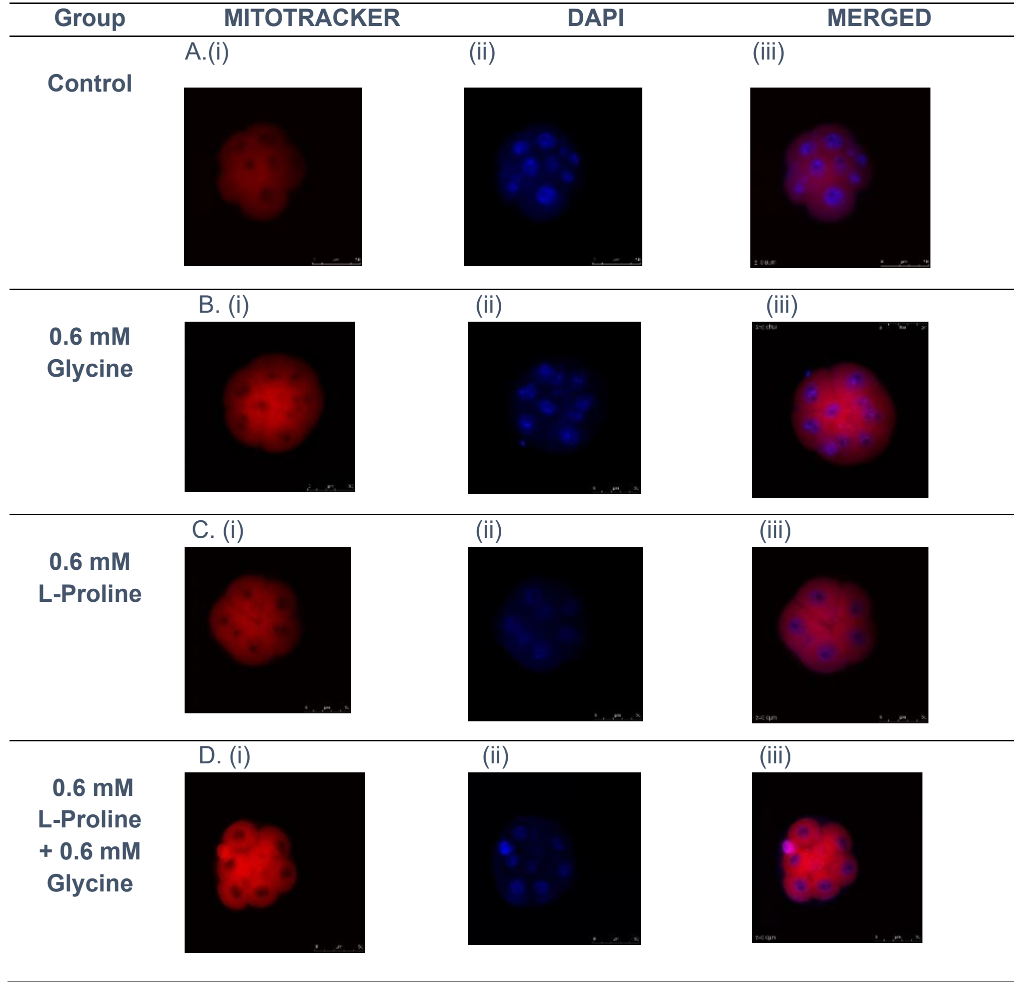

Figure 3. Fluorescent photomicrograph of 8-cell embryos showing intracellular mitochondrial contents (red) and nucleus (blue). Images viewed under 400x magnification. The scale bar for the images represents 25 μm

Representative microscopic images of mitochondrial distribution and intensity across all groups are shown in Figure 3. After culturing in vitro in different groups, the 8-cell embryos were stained with MitoTracker red to visualize homogeneously distributed mitochondria in the cytoplasm, and the color intensity indicates mitochondrial presence. From the study, Figure 3A shows that the staining in the control group embryo was less intense and more restricted to the periphery of the cytoplasm than in the embryo treated with plain L-Pro (Figure 3B). Both the embryos of plain Gly (Figure 3C) and those with combined Gly-L-Pro (Figure 3D) showed the strongest staining, indicating the intensity of mitochondria increased and was seen in the central parts of the cytoplasm. Such descriptions were associated with the mitochondrial fluorescence-intensity results shown in Figure 2.

The mitochondrial analysis in this study revealed that all treatment groups showed increased mitochondrial distribution and intensity, with the Gly-L-Pro combination exhibiting the highest intensity, followed by single Gly or L-Pro. Mitochondria have the task of generating the necessary energy, ATP to perform dynamic polymerization and precise attachment of spindle fibers required for cell differentiation and proliferation [27]. Studies on the role of amino acids in mitochondrial function have led to findings that treatment with Gly improves mitochondrial function and promotes cellular distribution [28]. In another study, L- Pro was found to be a potent exogenous substrate for mitochondrial metabolism that can prevent ATP loss [29]. It was also indicated that L-Pro plays an important role in maintaining ATP production when the mitochondrial complexes are impaired. As both amino acids demonstrated their strengths, the combined Gly-L-Pro group led to increased mitochondrial intensity and distribution. Such complementary roles of the two amino acids could have increased the number of active mitochondria and distributed them homogenously across embryonic cells. The embryos were able to proliferate and differentiate stably. The strongest intensity of the mitochondrial staining was observed in embryos supplemented with Gly-L-Pro and was consistent with the results of the study, which showed increased developmental competence, high blastocyst yields, and high hatching rate of blastocysts.

Measurement of ROS Level of 8-Cell Embryos in 0.6 Mm Glycine and L-Proline as Single and Combined Supplementation Preparations

For this study, 0.6 mM was chosen as the optimal concentration in both the individual and combined treatment groups. The results of fluorescence intensities produced by oxidized H2DCFDA in 8-cell embryos are shown in Figures 4 and 5. Although not significant, there was a decrease in ROS level in a single 0.6 mM Gly (25.23 ± 1.483 pixel 105) compared with the control group ($p > 0.05$). Meanwhile, the ROS level from the single L-Pro group [(42.41 ± 1.448 pixel 10^5) was found significantly higher than that in the control group (31.38 ± 1.966 pixel 10^5) ($p<0.01$), which in contrast with the antioxidant role of L-proline. This finding suggests that the effect of L-proline on embryonic redox regulation may be more complex than a simple reduction in oxidative stress [30]. L-proline may exert concentration-dependent effects that could alter mitochondrial metabolism and transiently increase ROS generation. Thus, L-proline alone may not have been sufficient to confer a net antioxidant effect [31].

However, the combined Gly-L-Pro group had a significantly lower ROS level (22.24 ± 1.542 pixel 105) than the control group ($p < 0.05$), but was not significantly different from the single 0.6 mM Gly group. Nonetheless, the results of this study show that the Gly-L-Pro combination can lower the ROS levels.

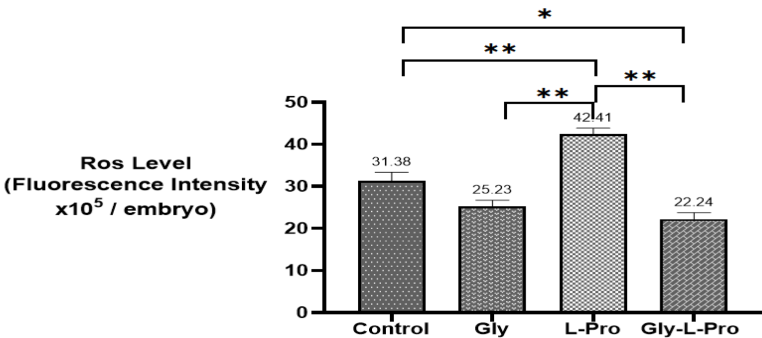

Figure 4. Level of ROS in 8-cell embryos con (control), Gly (0.6 mM Gly), L-Pro (0.6 mM L-Pro), and combined Gly-L-Pro (0.6 mM Gly + 0.6mM L-Pro) presented as fluorescence intensity (in pixel x 10^5). Data were presented as mean ± SEM to reflect the precision of the estimated group mean across biological replicates. Individual values were illustrated. * $p \leq 0.05$ and ** $p \leq 0.01$

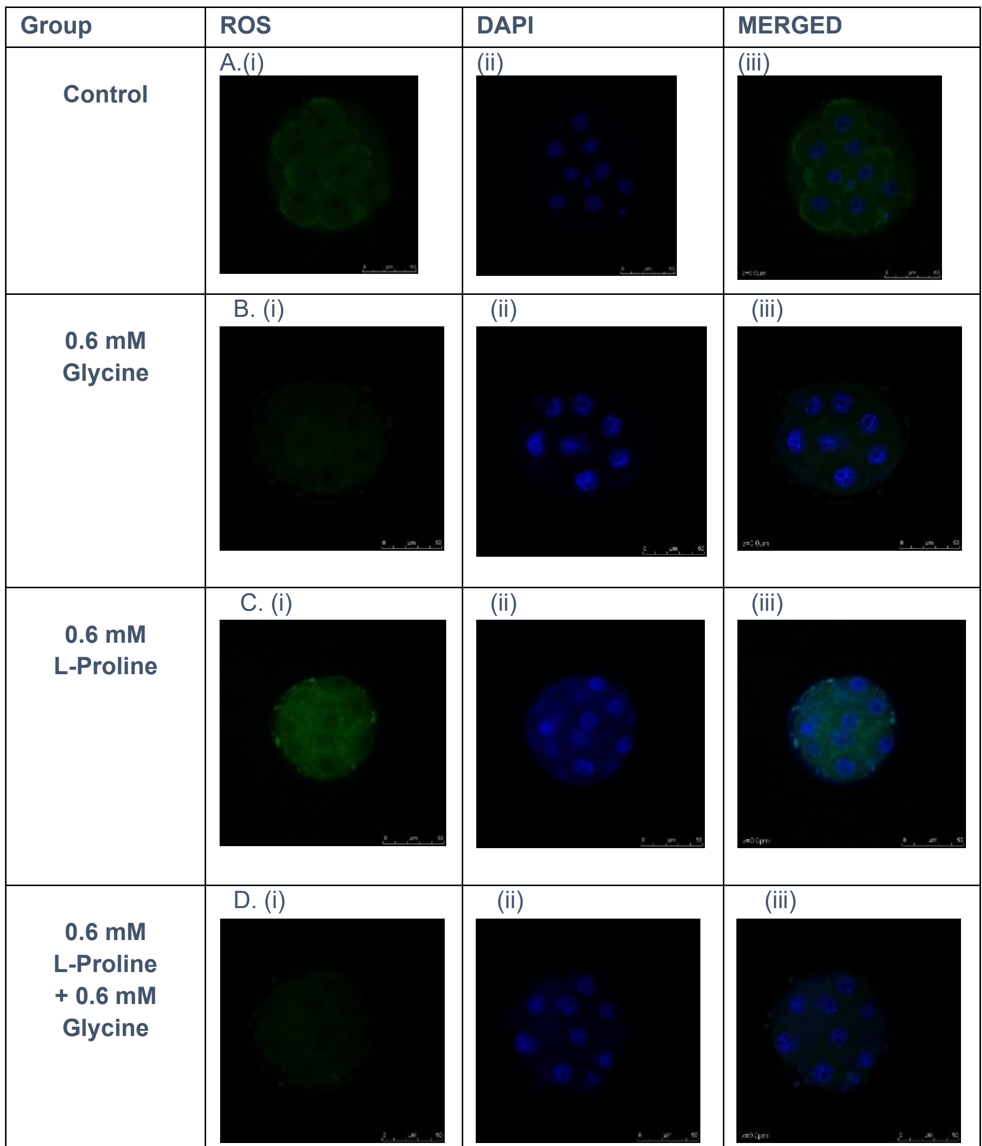

Figure 5. Fluorescent photomicrograph of 8-cell embryos with stained intracellular ROS (green) and nucleus (blue). Images viewed under 400 x magnification. The scale bar for the images represents 25 µm.

Another important property of Gly is its role as a buffer to maintain the pH level in the culture media by reducing the concentration of ammonia, which is a byproduct of cell metabolism and accumulates in embryo culture [32]. Although the presence of ammonia can promote the development of the mouse embryo [33] an increased concentration of ammonia can lead to toxicity and impair embryo growth [34]. Apart from ammonia, ROS are frequently produced during development by oxidative phosphorylation during ATP generation in the mitochondria [35]. Physiological concentrations of ROS are essential for pronuclear formation, first cleavage, and cell proliferation [36]. However, an excessive ROS has detrimental effects on embryo development, DNA damage, and protein oxidation [35], which ultimately leads to cell death by apoptosis [37]. In addition to cysteine and glutamine, glycine is also one of the precursors of glutathione, a potent endogenous antioxidant [50]. A previous study has shown that glutathione improves early development in bovine embryos [51] and in vitrified mouse embryos [52].

The results of this study were further confirmed by measuring ROS levels, which suggested that the presence of combined Gly-L-Pro significantly reduced ROS levels. This result suggests that the addition of Gly-L-Pro to the embryo culture medium reduces oxidative stress and may be involved in mitochondrial repair, providing mitochondrial functions such as cellular homeostasis and stress resistance [53]. The

scavenging of radical-free activity by Gly-L-Pro may have reduced the ROS content, thereby increasing the percentage of embryonic development and decreasing the number of abnormal cells.

Although the present findings provide evidence that amino acid supplementation may improve preimplantation embryo development under in vitro conditions, direct translation to human ART remains premature. Embryonic metabolism and developmental regulation are known to vary across species and even between strains, and such strain-specific differences may influence the response to culture conditions and nutrient supplementation. The current study was performed entirely in vitro. Therefore, another important limitation is that implantation potential, fetal development, and live birth outcomes were not evaluated. As a result, although the data support a beneficial effect during preimplantation development, the long-term viability and translational relevance of these embryos remain uncertain. Future studies should therefore include in vivo embryo transfer models, assessment of implantation and live birth, and validation in more clinically relevant systems before implications for human ART can be firmly established.

Conclusions

In conclusion, this study represents the first documented use of combined 0.6 mM Glycine and 0.6 mM L-Proline supplementation in embryo culture media. The results emphasise the importance of tailored nutritional supplementation for embryonic growth and viability and may offer new opportunities to improve fertility treatments in Assisted Reproductive Technology and developmental processes.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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