Dipeptide forms of glycine support mouse preimplantation embryo development in vitro and provide protection against high media osmolality

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Abstract

Purpose To examine potential benefits of dipeptide forms of amino acids for embryo culture by determining ability of dipeptide glycine forms to support embryo development, act as osmolytes, and reduce ammonia production.

Methods Frozen thawed 1-cell mouse embryos were cultured in media with varying osmolality with glycine and dipeptide forms of glycine and development assessed. Ammonia levels were measured in various media.

Results Dipeptide forms of glycine, alanyl- and glycyl-glycine, can support mouse embryo development in vitro. Additionally, dipeptide glycine can act as an organic osmolyte in developing embryos, permitting blastocyst formation in high osmolality media. Interestingly, as evidenced by decreased embryo development, dipeptides are not as efficient as osmolytes as their constituent individual amino acids. Dipeptide glycine produced less ammonia than glycine.

Conclusion Though dipeptides can provide osmoregulation in preimplantation embryos, efficacy may be lower than individual amino acids. The mechanism by which embryos

transport and utilize dipeptide amino acids remains to be identified.

Keywords Embryo · Blastocyst · Glycine · Dipeptide · Osmolyte · Ammonia

Introduction

Amino acids are important components of embryo culture media, serving as metabolic substrates and homeostatic regulators, where they can act as zwitterions to buffer pH, antioxidants, and organic osmolytes [1]. Because even brief exposure of mouse zygotes to media lacking amino acids can impair development [2], most in vitro fertilization (IVF) culture media now contain some assortment of the molecules. On the other hand, not all amino acids are necessarily beneficial or required for embryo development. Experiments conducted in mouse embryos utilizing commercially available mixtures of amino acids known as Eagle's 'essential' and 'non-essential' amino acids determined a preferential exposure sequence that improved embryo development and viability following transfer, which entailed addition of non-essential amino acids with glutamine during the first 2 days of culture, followed by inclusion of all 20 amino acids during the following 2–3 days [3]. Similarly, benefits and detriments of individual amino acids have been shown in various studies in a variety of species. For example, during culture of hamster 1-cell embryos, glutamine, taurine and glycine appeared to be superior to other amino acids studied [4]. Identification of key individual amino acids is important to avoid possible inclusion of amino acids that may convey a negative effect.

One particular amino acid that appears important for embryo homeostasis is glycine. Preimplantation embryos are sensitive to osmolality, with media of $> \sim 300$ mOsm

Capsule Dipeptide amino acids may offer a means of improving in vitro embryo culture. However, information regarding their use for embryo culture is scarce. We examine the ability of dipeptide forms of glycine to support embryo development and act as osmolytes. Future studies are needed to determine how embryos transport/utilize dipeptide amino acids.

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impairing development [5–7]. Unfortunately, detrimental osmolality shifts can be produced from common laboratory media preparation procedures [8]. Glycine is a potent osmolyte that provides protective effects to developing embryos against detrimental rises in media osmolality [5, 9, 10]. The Na⁺ and Cl[−]-dependent GLYT transporter used by glycine is found in cleavage stage embryos in the mouse [11–14], and also in humans [15].

An additional concern with amino acids, especially during cleavage stage development, is their breakdown in culture and resulting production of ammonia, which may impact subsequent embryo development and health of resulting offspring [16–20]. However, it should be noted that the relevance of this phenomenon in the context of conditions used to culture embryos is questionable [21]. Regardless, one method to minimize the potential risk of ammonia production from amino acids is by defining specific physiologic roles of amino acids and including only key amino acids in embryo culture media. Another method utilized to combat ammonia production is through use of more stable dipeptide amino acids [22].

Various dipeptide amino acids have been shown to be adequate substitutes for their corresponding essential amino acids, and can sustain growth and multiplication rates of somatic cells lines, including mouse fibroblast and HeLa cells, for several weeks when using the same molar concentrations [23]. In the case of glutamine, however, early rounds of cell division in a murine hybridoma cell line during the lag period were reported to be slowed when cultured in the presence of dipeptides compared to their single amino acid counterparts [24], which questions efficacy of dipeptides and cellular utilization mechanisms for the compounds. Though dipeptide forms of the amino acid glutamine, such as alanyl-glutamine [19] or glycyl-glutamine [25, 26], have been utilized successfully for embryo culture, information on the use of other dipeptides in embryo culture is lacking. Furthermore, no studies exist verifying that dipeptide amino acids are able to provide the same known physiologic roles as the corresponding single amino acids, like providing protection against high media osmolality. Therefore, the objective of this study was to examine the impact of a previously unstudied dipeptide in mouse embryos, focusing on a known beneficial amino acid with established functions. We examined the impact of dipeptide forms of glycine on mouse embryo development *in vitro*, and determined their ability to act as osmolytes and protect development against high media osmolality.

Materials and methods

All ingredients used in media formulation were supplied by Sigma (St. Louis, MO), unless otherwise indicated. Frozen

thawed 1-cell mouse embryos (B6C3F-1 x B6D2F-1; Vitro-life, Englewood, CO) were used for all developmental experiments.

Osmolality and embryo culture

Human Tubal Fluid medium (HTF) was formulated for embryo development studies [27]. Frozen thawed 1-cell mouse embryos were cultured continuously in groups of 10–12 in 500ul media covered with 300ul mineral oil (Irvine Scientific, Santa Ana, CA) in 4-well dishes for 96 h in ~6% CO₂ in air with Thermo Forma incubators. pH of all media were kept between 7.27 and 7.32. Positive control media had an osmolality of 285–290 mOsm. Osmolality of media for various studies was adjusted by altering NaCl levels. Development was assessed at various time points to create a sensitive assay to determine impact of osmolality and dipeptides.

Dipeptides and embryo development

To determine impact of dipeptide glycine forms and potential toxicity, embryo development was first evaluated in HTF+5% Serum Substitute Supplement (SSS, Irvine Scientific) supplemented with 1.0 mM alanyl-glycine or glycyl-glycine. Positive controls consisted of 1.0 mM glycine. The medium was primarily selected due to the lack of amino acids, but also because it is a common medium used in human embryo culture. Additionally, protein source and concentration are also those commonly used in human embryo culture. Concentrations of glycine and dipeptide forms were selected based on apparent maximum effective concentration of glycine in mouse embryos [5].

To determine protective effects of glycine forms against osmotic stress, embryos were cultured in 310 mOsm HTF+5% SSS with no amino acid, 1.0 mM glycine, 1.0 mM glycyl-glycine, or 1.0 mM alanyl-glycine. This osmolality value was determined to be appropriate, as it was previously shown to be a midpoint elevated osmolality achieved under a variety of preparation conditions in an IVF laboratory [8]. Additionally, 310–320 mOsm was previously shown to inhibit embryo development [8], but also provides a sensitive system in which development can still be rescued [5]. Subsequently, 1 mM alanyl-glycine was compared to 1 mM alanine+1 mM glycine to examine efficacy of dipeptides compared to component individual amino acids in supporting embryo development in high osmolality media (320 mOsm). Total blastocyst cell number were then counted following Hoescht staining. Developmental data were collected over three to six replicates, with 10–12 embryos per treatment per replicate, and statistical differences determined using ANOVA followed by Tukey analysis.

Ammonia assay

To determine if dipeptide forms of glycine offer the advantage of reduced ammonia production compared to glycine, an ammonia assay kit (Abcam, Cambridge, MA) was utilized to detect ammonia production in culture media following 72 h incubation at 37°C, mimicking what might be experienced within a human embryology lab. 100x (100 mM) stocks of glycine, glycyl-glycine and alanyl-glycine were prepared and incubated. Reagents were mixed according to the kit protocol. Briefly, ammonia from samples acts as a substrate which reacts with the supplied enzymes to form a product that reacts with the OxiRed probe to generate color, which is then read on a plate reader (Lambda max=570 nm). The kit can detect 1 nmol (~20 μ M) of ammonia, and is more sensitive than using a NADPH based ammonia assay. Standard curve samples, controls, and assay samples were all performed in triplicate and data analyzed using ANOVA followed by Tukey analysis. Data is presented as the mean $\pm$ SEM over control media, which contained no amino acids.

Results

Dipeptide glycine and embryo development

Examining the impact of various dipeptides in media with normal osmolality (~290 mOsm) over three replicates, no significant differences were observed in rates of early cleavage at 6 h (46.7% $\pm$ 8.8, 53.3% $\pm$ 8.8, 46.7% $\pm$ 12.0), cleavage >2-cell at 30 h (63.3% $\pm$ 8.8 vs.73.3% $\pm$ 12.0 vs.60.0% $\pm$ 15.3), development $\geq$ 8-cell at 48 h (90% $\pm$ 5.8 vs. 93.3% $\pm$ 3.3 vs.86.7% $\pm$ 3.3), early blastocyst formation at 72 h (56.7% $\pm$ 3.3 vs. 60.0% $\pm$ 15.3 vs. 40.0% $\pm$ 11.5), total blastocyst formation at 96 h (76.7% $\pm$ 3.3 vs. 86.7% $\pm$ 8.8 vs. 83.35% $\pm$ 8.8), or rates of blastocyst hatching (63.3% $\pm$ 6.7 vs.56.7% $\pm$ 8.8 vs.50.0% $\pm$ 5.8), between glycine, glycyl-glycine or alanyl-glycine, respectively (Fig. 1). Therefore, all forms of glycine examined appeared suitable for embryo culture.

In examining impact of various dipeptides on embryo development in hyperosmolar (310 mOsm) media over 4 replicates, negative controls without glycine yielded 33.3% $\pm$ 3.0 early cleavage at 6 h, 12.1% $\pm$ 6.4 cleavage >2-cell at 30 h, 30.3% $\pm$ 8.0 development $\geq$ 8-cell at 48 h, 6.1% $\pm$ 6.1 early blastocyst development at 72 h, 51.3% $\pm$ 18.3 total blastocyst formation at 96 h and 6.1% $\pm$ 6.1 blastocyst hatching at 96 h. In this group, the majority of embryos that were not at the time-appropriate stage of development appeared to arrest between the 2-4cell stage and underwent subsequent degradation during extended culture periods. Positive control media (290 mOsm) yielded, 39.4% $\pm$ 6.1 early cleavage

Fig. 1 One-cell mouse embryos in culture media with normal osmolality (~290 mOsm), were grown in the presence of 1 mM glycine, 1 mM alanyl-glycine and 1 mM glycyl-glycine to determine if dipeptides could support embryo development. No differences in development at any time point examined were observed, indicating no overt toxic effects of dipeptide forms of glycine

at 6 h, 45.5 $\pm$ 10.5% early cleavage at 30 h, 90.9% $\pm$ 5.3 development $\geq$ 8-cell at 48 h, 48.55% $\pm$ 3.0 early blastocyst development at 72 h, 93.9% $\pm$ 3.0 total blastocyst formation at 96 h and 33.4% $\pm$ 8.0 blastocyst hatching at 96 h. Media supplemented with glycine, glycyl-glycine and alanyl-glycine yielded similar rates of early cleavage at 6 h (39.4% $\pm$ 8.0, 42.4% $\pm$ 8.0, 42.4% $\pm$ 13.2), cleavage >2-cell at 30 h (51.5% $\pm$ 6.0 vs.54.6% $\pm$ 13.9 vs.54.6% $\pm$ 22.9), development $\geq$ 8-cell at 48 h (81.8% $\pm$ 9.1 vs. 69.7% $\pm$ 21.2 vs.93.9% $\pm$ 6.1), early blastocyst formation at 72 h (45.5% $\pm$ 21.0 vs. 39.4% $\pm$ 18.4 vs. 60.6% $\pm$ 18.4), total blastocyst formation at 96 h (82.1% $\pm$ 13.6 vs. 75.8% $\pm$ 15.1 vs. 90.9% $\pm$ 0.0), or rates of blastocyst hatching (42.4% $\pm$ 8.0 vs.48.5% $\pm$ 18.4 vs.48.5% $\pm$ 8.0), respectively. All forms of glycine examined rescue embryo development in media with high osmolality by supporting significantly higher rates of embryo development compared to negative controls beginning at the 30 h time point and continuing at 48, 72 and 96 h ($p < 0.05$) (Fig. 2). No significant differences were apparent at any time point between forms of glycine tested or with positive controls.

To determine how dipeptide amino acids compare to their individual amino acid counterparts in regard to ability to support embryo development and act as efficient osmolytes, dose compensation studies were performed over six replicates. Because prior findings revealed alanyl-glycine appeared to be a more efficient osmolyte than glycyl-glycine, 1 mM alanyl-glycine was compared to 1 mM alanine + 1 mM glycine to support embryo development in a high osmolality media. Additionally, a slightly higher osmolality of 320 mOsm was utilized to create a more sensitive system and improve ability to determine osmolyte efficacy. Data indicate use of individual amino acids serve as more potent osmolytes compared to the dipeptide. Though not statistically significant, alanine + glycine consistently

Fig. 2 a Ability of dipeptide forms of glycine to act as organic osmolytes to developing embryos was examined. One-cell mouse embryos were grown in high osmolality media (~310 mOsm) in the presence of known osmolyte 1 mM glycine, as well as 1 mM alanyl-glycine or 1 mM glycyl-glycine. Positive controls consisted of 290 mOsm media, while negative controls consisted of 310 mOsm media with no amino acid/dipeptide supplementation. Embryo development was examined over 96 h and both dipeptide forms of glycine were able to rescue embryos development in high osmolality media, providing similar results to positive controls and glycine alone at various time points. **b** Further analysis of type of blastocyst formation at 96 h was performed. Different superscripts within a time point/represents a statistically significant different, $p < 0.05$

yielded faster rates of development at 30 and 48 h compared to the dipeptide. Additionally, though not statistically significant, alanine + glycine yielded higher rates of blastocyst formation at 72 h compared to the dipeptide ($51.1\% \pm 11.7$ vs. 25.05 ± 8.2) and total blastocyst formation at 96 ($81.16.4\% \pm 6.5$ vs. $67.8\% \pm 6.6$) (Fig. 3a). More detailed examination on type of blastocyst formation at 96 h was then examined, and though no differences in rate of early blastocyst, full blastocyst, or expanded blastocyst development was observed, alanine+glycine yielded significantly higher rates of blastocyst hatching at 96 h compared to the dipeptide ($41.7\% \pm 9.1$ vs. $9.4\% \pm 4.9$) (Fig. 3b). While use of individual amino acids yielded similar rates of development at all time points examined compared to positive controls (290 mOsm), the dipeptide yielded significantly lower rates of total blastocyst formation and hatching rates at 96 h (Fig. 3a, b). Finally, though blastocysts formed in 290 mOsm media yielded significantly higher cell numbers compared to 320 mOsm media (60.2 ± 4.2 vs. 36.2 ± 2.0), no significant differences were apparent between alanine+glycine

(56.9 ± 5.3) and the dipeptide (51.8 ± 3.6) or between other treatments (Fig. 4).

Ammonia assay

Both alanyl- and glycyl-glycine at 100 mM produced less ammonia than 100 mM glycine, though overall ammonia production for all of the amino acids studied was extremely low, and likely not a concern in regard to producing sufficient amounts to convey a negative impact on embryo development (Fig. 5).

Discussion

Dipeptide amino acids, in the form of glutamine, are widely used in human IVF culture medium. However, efficacy of dipeptides, and their utilization by developing preimplantation embryos, has received very little examination. Though a few studies exist showing dipeptides, such as alanyl- and

Fig. 3 **a** Efficacy of the dipeptide 1 mM alanyl-glycine to serve as an organic osmolyte was assessed by comparing embryo development in high osmolality media (320 mOsm) to media supplement with component individual amino acids (1 mM alanine+1 mM glycine). Both the dipeptide and individual amino acids supported higher rates of embryo development compared to negative controls of 320 mOsm at various time points, yielding similar results to each other. However, though not statistically significant, development in the presence of the dipeptide was consistently lower than when using individual amino acids. Furthermore, dipeptide use yielded significantly lower rates of blastocyst formation at 96 h compared to positive controls (290 mOsm). **b** Further

examination of type of blastocyst formation/expansion at 96 h was performed. Though no differences were apparent between treatments in regard to early to full blastocyst expansion, both the dipeptide and individual amino acids yielded significantly higher rates of expanded blastocyst formation compared to 320 mOsm, similar to rates of 290 mOsm. Interestingly, individual amino acids supported significantly higher rates of hatching compared to 320 mOsm and the dipeptide, similar to those obtained in positive control 290mosm. Different superscripts within a time point/represents a statistically significant different, $p < 0.05$

glycyl-glutamine, can support embryo development in normal culture media [16, 25, 26, 28], no studies exist examining efficacy of dipeptides in supporting specific physiologic roles of amino acids in embryos, such as acting as osmolytes [5, 9, 10, 29–31]. We have reported here for the first time that the dipeptides alanyl-glycine and glycyl-glycine can function as organic osmolytes in developing

embryos; however, use of individual amino acids counterparts function more efficiently.

Various amino acids can serve as osmolytes to develop embryos at varying stages of development. Glycine is perhaps the most potent osmolyte in early stage preimplantation embryos and provides a protective mechanism to rescue embryo development when cultured in media with

Fig. 4 As an added measure of blastocyst quality and marker of osmolyte efficacy, total blastocyst cell number were determined following 96 h of culture in 320 mOsm media supplemented with 1 mM alanyl-glycine or 1 mM alanine+1 mM glycine. Negative controls consisted of 320 mM media and positive controls consisted of 290 mOsm media. Though blastocysts grown in positive control media yielded significantly higher cell numbers compared to negative controls, no difference were observed between any other treatment ($p < 0.05$)

detrimentally high osmolality. Glycine was able to rescue fresh mouse embryo development when cultured from the 1-cell stage to blastocyst in 310–330 mOsm KSOM supplemented with PVP [5]. This osmo-regulatory role is dependent upon glycine transport into the cleavage stage embryos by the GLYT1 transporter system [9, 11, 12, 14, 32–34]. In post-compaction mouse embryos, glycine transport can utilize both the GLYT1 and B⁺,0 transporter [31, 35]. This is relevant because we have now demonstrated that dipeptide forms of glycine (alanyl- and glycyl-glycine) can also function as osmolytes in preimplantation mouse embryos. However, it is unknown how these dipeptides are specifically transported into the cell or whether transport differs between cell stages. Answers to these questions requires insight into how preimplantation embryos process dipeptide amino acids.

In other cell systems, dipeptides may be hydrolyzed outside the cell into constituent amino acids before uptake by known amino acid transporters and utilization within the cell. Indeed, in a murine hybridoma cell line, peptidase activity was found in the culture media, secreted from the cells, that likely hydrolyzed dipeptide forms of glutamine [22]. A similar peptidase may be secreted from embryos. Certainly, trophoblast cells have membrane-bound peptidases [36, 37], and perhaps similar enzymes are present in cleavage stage embryos that may hydrolyze dipeptides. If this is the case, embryo density and dynamic culture systems are obvious factors to consider when including dipeptides in culture media. Alternatively, specialized dipeptide transporters may exist. The intestinal proton-coupled amino acid transporter SLC36A1 functions in Caco-2 cell monolayers and transports Gly-Gly and other glycine dipeptide mimetics [38]. The dipeptide glycyl-glutamine appears to be taken up

in rat choroid plexus epithelial cells by the PEPT2 transporter, before being hydrolyzed within the cell [39]. Importantly, these studies have shown dipeptide uptake was inhibited by the presence of other dipeptides in the media, but unaffected by individual amino acids [39]. Thus, if similar systems operate in embryos, inclusion of multiple dipeptides in culture media may be problematic. Also, while glycyl-glycine was transported by SLC36A1, glycyl-alanine was not [38]. While this particular transporter may not necessarily be functioning in preimplantation embryos, it still suggests that glycyl-glycine and alanyl-glycine could be handled in differing fashions by embryos, and possibly explain the slight differences observed in this study between the dipeptides. This may also be relevant to current systems utilizing either alanyl- or glycyl-glutamine and may explain observed differences [25]. It should be mentioned that initial studies also demonstrated no difference in ability to support embryo development in high osmolality media between alanyl-glycine and glycyl-alanine (data not shown).

This question of dipeptide utilization by embryos is highlighted by findings in our study, which demonstrated that alanine supplemented with glycine provides superior osmo-protection compared to the dipeptide alanyl-glycine. This may suggest superior uptake and utilization of individual amino acids by embryos versus the dipeptide. One possible explanation could be increased time needed to cleave the dipeptide outside the cell, before individual amino acids can be transported inside the cell to act as osmolytes. Alternatively, dipeptide transporters may operate at a lower efficiency. This theory is supported by findings from studies in somatic cells that demonstrate reduced cell numbers during the lag phase when grown in the presence of dipeptide glutamine compared to glutamine alone [22], as well as a

Fig. 5 Use of dipeptide amino acids offers a potential approach to reduce concern with ammonia production and resulting negative effects on the developing embryo and fetus. Both alanyl- and glycyl-glycine yield significantly lower amount of ammonia following 72 h of culture at 37°C compared to glycine, though overall amounts produced by all treatments was minimal. Data are presented as an increase/decrease compared to control media with no amino acids/dipeptides. Different superscripts between treatments represent a statistically significant different, $p < 0.05$

required 48-h adaptation period needed for cells to adapt to the presence of dipeptide glutamine, compared to glutamine alone, to achieved appropriate growth [40].

In addition to how dipeptides are utilized and transported by embryos, another important consideration in determining the true impact of dipeptides is resolving whether benefit is conveyed from the dipeptide itself, or simply through the actions of the constituent amino acids. For example, a prior study demonstrating that glycyl-glutamine was superior to alanyl-glutamine in supporting mouse embryo development [25] could simply be due to the fact that glycine is a more beneficial amino acid than alanine. Indeed, glycine has been shown to be superior in hamster embryos and is a superior osmolyte compared to alanine [4, 31]. In our study, alanyl-glycine supported slightly higher embryo development in high osmolality media compared to glycyl-glycine. Though glycine has been shown to be a superior osmolyte compared to alanine in previous studies [31], as well as in our lab (data not shown), this apparent contradiction may be explained by 1 mM glycyl-glycyl possibly saturating the GLY1 transporter system and limiting ability of the cell to cope with high osmotic stress. Glycine concentrations around 1 mM appear to offer maximal protection against high media osmolality in the mouse [5]. Supporting this theory, we also demonstrated no improvement in embryo development in high osmolality media when comparing 1 mM or 2 mM glycine to glycyl-glycine (data not shown). The benefit of alanyl-glycine may be that it uses separate transporter systems; those used to transport alanine ($B^{0,+}$ transporter)[35, 41, 42], as well as those used to transport glycine. This mechanism would obviously require peptidase action and external hydrolysis of the dipeptide. Indeed, alanine and glycine have been shown to have synergistic effects in improving bovine embryo development [43, 44] and may help explain slightly improved results obtained with the alanyl-glycine dipeptide.

One of the primary rationales promoting the use of dipeptide amino acids, like glutamine, in IVF is due to increased stability at higher temperatures and lower ammonium production. In one of the first reports on the topic, ammonia production in two commercial embryo culture media supplemented with an array of amino acids, including alanyl-glutamine, yielded lower ammonium levels compared to other media without alanyl-glutamine [16]. However, ammonium levels in the first 48 h of culture in these media with an array of several amino acids along with the dipeptide were also lower than that of a commercial medium with 0.05 mM taurine as its only amino acid. Thus, results should be interpreted with caution and actual quantification and assessment of the benefit of dipeptide glutamine in embryo culture media in respect to ammonium remains to be determined. We therefore measured ammonium production in our study of dipeptide glycine forms using an extremely sensitive assay. Despite increased assay sensitivity,

we were still required to use 100X stocks of glycine dipeptides to permit measurement. Following incubation, we showed that, though alanyl- and glycyl-glycine produce less ammonium than glycine, amounts produced from glycine are low enough to likely have no impact on resulting embryo development. Thus, in respect to reduced concern of ammonia production, there is likely no added benefit of using dipeptide forms of glycine.

In conclusion, use of dipeptide forms of amino acids, such as glutamine, are now widely used in human IVF. Whether use of other dipeptides may offer advantages is unknown. We have examined dipeptide forms of glycine, a widely used amino acid and known embryo osmolyte, and demonstrated that reduction of ammonia production is likely insufficient rationale to adopt its use. Perhaps of more concern is the efficacy with which embryos can utilize dipeptides, as use of the component amino acids in the dipeptides provide superior osmoprotection compared to the corresponding dipeptide. This calls into question how embryos utilize and transport dipeptide amino acids and presents an important, unanswered question as to whether currently used dipeptides are appropriate and if use of other dipeptides during embryo culture may or may not be warranted.

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