The Impact of Two Embryo Culture Media, SOF and Commercial BO, on Pre- and Post-Implantation Development of Cloned Sannen Goat Embryos

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Research

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Abstract

Background: The ingredients of embryo culture media developed by different companies are disclosed. Thus, it is impossible to determine which ingredients might be responsible for differences in pre-and post-implantation embryo development. To address this gap, we performed an experiment to compare two embryo culture media, namely, SOF and commercial BO, on pre- and post-implantation development of cloned Sannen goat embryos. Cumulus oocyte complexes derived from slaughterhouse ovaries were used for in vitro embryo production. In vitro development of IVF, parthenogenetic and SCNT embryos were assessed in both BO and SOF media. The expression of 16 genes, including AKT, OCT4, SOX2, BMPR1, FGFR4, CDC25, CDX2, GCN5, PCAF, FOXD3, SMAD5, FZD, LIFR1, CTNNB, ERK1, and IFNT, belonging to 7 important pathways, i.e. pluripotency, FGF, TGFβ, cell cycle and proliferation, histone transferase, trophoderm, and WNT, were examined in the goat SCNT and IVF blastocysts from both BO and SOF media.

Results: The blastocyst rate in BO medium was significantly higher than that of the SOF medium in SCNT embryos (P < 0.05). All of the genes examined showed increased expression levels in SCNT embryos compared to IVF embryos. In the IVF group, OCT4, BMPR1, and GCN5 showed significantly higher expression in the SOF medium compared to the BO medium. In this group, AKT, FGFR4, SOX2 showed significantly lower expression in the SOF medium compared to the BO medium. In the SCNT group, FGFR4, GCN5, FZD, CTNNB, BMPR1, and FGFR4 showed significantly higher expression in SOF medium compared to BO medium. In vivo development did not differ significantly between the two groups.

Conclusions: Based on these results, we concluded that the limited information available on the allocations of ICM and TE cells in SCNT embryos and embryo-specific gene expression may be the major drawback IVC medium and an impediment to successful animal cloning.

Background

Lesson from the observation and investigation of normal physiological systems, especially the female reproductive system, has resulted in the formulation of media to improve in vitro culture conditions and suit the embryo’s requirements [1]. Based on the literature, it is possible to have a rough estimate of the performance of each media type regarding pre- and post-implantation competence [2]. However, it must be borne in mind that in addition to the medium components [3] and embryo metabolism [4, 5], factors influencing the culture medium condition [6, 7], including air quality, temperature, and humidity, can also impact the developmental competence [8].

Regarding human embryo culture, numerous commercial culture media are available in the market [9–11]. Although the efficiency of some of these media has been assessed using a mouse model, there are a limited number of studies which have compared these commercially available media [12–13]. A quick review of the literature showed that the introduction of in vitro fertilization (IVF) for the treatment of infertility was associated with single-step culture media and cell culture media, such as Ham's F10,
KSOMaa, Bracket-Oliphant (BO) [8, 14–18]. However, further research resulted in the introduction of sequential culture media, which were based on the composition of human tubal fluid [19]. Then, with the introduction of time-lapse technology in embryology and some new clinical trials, single-step culture media gained renewed attention and were started to be used routinely in embryo culture media, especially when time-lapse technology was used to monitor embryos [20–23]. Single-step media can also be useful in the zona-free somatic cell nuclear transfer (SCNT) method, in which the embryos should be cultured in an undisturbed environment to support the development of SCNT embryos to the blastocyst stage.

The efficiency of SCNT in biotechnology [20], biomedicine, stem cells [24], transgenic animal production [25], conservation of endangered species, and breeding has attracted considerable attention in recent years [26]. However, despite the significant improvements made to enhance the reprogramming efficiency of SCNT, the technical efficiency and high throughput of SCNT has limited its application and reduced its success rate compared to IVF [27–29]. However, only a limited number of studies have focused on the type of culture media and their effect on the efficiency of SCNT [30–32]. In a previous study, we showed that sequential G-series culture media, despite resulting in a high blastocyst formation rate, had a limited effect on oocyte developmental competence compared to that of sequential synthetic oviductal fluid (SOF) media [33]. In the light of the above information, this study was designed to compare the impact of SOF medium with that of commercial BO medium, commonly used for in vitro production of farm animals, using caprine SCNT embryos.

**Methods**

Chemicals and media were purchased from Sigma (St. Louis, MO, USA) and Gibco (BRL, Grand Island, NY, USA).

**Oocyte in vitro maturation**

Briefly, the ovaries of Bakhtiari goat were obtained from a slaughterhouse in Isfahan. Following the dissection of ovaries around 2–4 pm, they were transferred to thermos flasks, containing normal saline with 2X antibiotics (0.1 mg/mL streptomycin, 100 IU/mL penicillin G potassium), and maintained at 15–17 °C until they were transferred to the laboratory by 6 pm. Immediately, the ovaries were washed, trimmed, and stored at 11–12 °C until the cumulus oocyte complexes (COCs) were harvested the day after at 9 am.

The in vitro maturation (IVM) was performed as described in our previous study [26]. Briefly, the follicular content was aspirated from 2–6 mm follicles using a 20-gauge needle attached to a vacuum pump. Only COCs with homogeneous cytoplasm and at least three layers of compact cumulus cells were isolated for IVM. The COCs were cultured in 50 µl droplets of maturation medium, containing tissue culture medium 199 (TCM-199) + 10% FBS (fetal bovine serum) supplemented with 10 µg/ml FSH (Follicle-stimulating hormone, sigma F8174), 10 µg/ml LH (Luteinizing hormone, sigma L5269), 100 mM 17-beta-estradiol, 0.1 mM cysteamine, 10 ng/ml EGF (epidermal growth factor), and 100 ng/ml IGF1 (insulin-like growth factor 1), under mineral oil for 20 hour at 38.5 °C, 5% CO2, in maximum humidified air.
**Somatic cell nuclear transfer**

We used a variant of SCNT technology which is called handmade cloning (HMC). After denudation of cumulus cells (using 300 IU/ml hyaluronidase) and the removal of zona pellucida with 5 mg/ml pronase for few seconds), a manual method of oocyte enucleation with the aid of a fine pulled Pasteur pipette was used to enucleate the oocytes [34]. Briefly, zona-free oocytes were incubated in TCM supplemented with 4 µg/ml demecolcine for 20 min at 38.5 °C. Then, a cytoplasmic protrusion, containing MII spindle, was removed by a manual pipette. For nuclear replacement, enucleated oocytes were transferred to dishes containing droplets of H-TCM supplemented with 10 mg/ml phytohemagglutinin; then, single fibroblast cells were attached to the membrane of the enucleated oocytes [34]. Subsequently, the couplets in fusion buffer free of Ca+2 and Mg+2 (290 mOsm) were electrofused using sinusoidal electric current (1.7 Kv/cm) for 10 sec, followed by two direct currents (1.75 kV/cm for 30 μsec and 1 sec delay). After 30 min, the reconstructed oocytes were activated with 5 µM ionomycin for 1 min, followed by 2 h of incubation with 2mM 6-dimethylaminopurine (6-DAMP) (Lan et al., 2005). Afterward, the activated reconstructed oocytes were cultured inside the wells containing SOF (synthetic oviductal uid) medium or BO (Bracket-Oliphant) medium and incubated for 7 days under mineral oil at 38.5°C, 5% CO2, 5% O2, and humidified air [34]. Grade 1 and 2 blastocysts were selected for embryo transfer.

**Selection of genes set**

In order to select the genes that could predominantly be involved in the regulation of early embryonic development and pluripotency and due to the lack of sufficient data on the goat species, we followed the same strategy described by McGraw et al. [35]. In brief, we sought for the related information using gene expression databases that profiled gene expression and gene ontologies (GOs) in both human and mouse embryos and ESCs (Table S.1). To be considered as a potential candidate, the genes had to be commonly present in ESCs and either oocyte or blastocyst and play critical roles in the transcription regulation, pluripotency, and differentiation. This survey provided a list of 17 genes, including AKT, OCT4, SOX2, BMPR1, FGFR4, CDC25, CDX2, GCN5, PCAF, FOXD3, SMAD5, FZD, LIFR1, CTNNB, ERK1, and IFNT. The main functions, GOs, and null alleles of each gene are summarized in Table S.1. Due to the lack of any previous report or database regarding the gene sequences of many of the abovementioned genes, the primers were designed based on the conserved regions of these markers in bovine, ovine, human, and mouse sequences. Subsequently, specific primers were designed from these recognized sequences (Table 2).
| Gene | Primer sequence (5’-3’) | Length of PCR product | TM |
|------|-------------------------|-----------------------|----|
| AKT  | F:CCCTTAACAACTTCCTCTG   | 98                    | 60 |
|      | R:GAATGACGAAGGTATTGG    |                       |    |
| Oct4 | F:AGAAGGGCACAACGTCAAGC  | 96                    | 56 |
|      | R:GAATGGGACCAGAAGGTACAGA |                      |    |
| Sox2 | F:GCCGCCGATGATTGTTAT    | 182                   | 54 |
|      | R:AGAGAGAAAGAAAGGGAGAGAA |                      |    |
| BMPR1| F:TGTTCGTCTGTTCTCAT     | 116                   | 58 |
|      | R:GGTGCTAAGGGTACTCC     |                       |    |
| FGFR4| F:GCTGACTGGYAGGAAAGG   | 193                   | 56 |
|      | R:AGTGCGCTGAGCACATCG    |                       |    |
| CDC25| F:TGGCAAGCGTTTTACGT    | 119                   | 58 |
|      | R:GGTAGTGGAGTTTGGGTA    |                       |    |
| CDX2 | F:CCCCAAGTGAAAACCAG    | 144                   | 53 |
|      | R:TGAGAGCCCCAGTGTG     |                       |    |
| GCN5 | F:ACTCACCTGATAACCCAC   | 174                   | 54 |
|      | R:TGTGCACCCCTCGTAG     |                       |    |
| PCAF | F:ACGAACAACTGCAAGGGCTATG| 246                   | 60 |
|      | R:CAGAGAACTCGTGTATGGG  |                       |    |
| Gene  | Forward Primer       | Reverse Primer       | Length | AUC |
|-------|----------------------|----------------------|--------|-----|
| FOXD3 | F:AGAGCCCGCA          | R:GGTCCAGTAG          | 182    | 59  |
|       | GAAGAAGC              | TTGCCCTTTG            |        |     |
| SMAD5 | F:ATTATGCCAAG         | R:GTCTGTGAAT          | 136    | 60  |
|       | TATATCCA              | CCATCTCAC             |        |     |
| FZD   | F:ATTGCTGCTA          | R:TTAGTCTGGT          | 89     | 59  |
|       | CCTTTTAC              | TGTTTCATT             |        |     |
| LIFR  | F:CGGATTCCGT          | R:AATGCGTTCT          | 117    | 56  |
|       | TTGTTACT              | GTGGTTAA              |        |     |
| CTNNB | F:TGGCTATTACA         | R:GGTCCCTATTA         | 160    | 54  |
|       | ACAGATT               | TATTCAC               |        |     |
| ERK   | F:GCTAATTCACC         | R:ACGATATAAGG         | 204    | 58  |
|       | TGGAGAT               | CGAGTTTG              |        |     |
| IFNT  | F:AGAATCCGTCT         | R:TCAGTCAACG          | 129    | 54  |
|       | CTACCTG               | AGAACCAC              |        |     |

Note. The measurements are ordered by decreasing AUC.

Table 2
Primers Used for Real-Time PCR Experiment

**Gene expression analysis**

RNeasy Micro Kit (Qiagen®, Germany) was used to extract total RNA from blastocysts. The extracted RNA was used for first-strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Germany). The cDNA synthesis reaction contained 1 µL random hexamer primer, 1 µl RNase inhibitor, 4 µl 5x reaction buffer, 2 µl dNTP, and 1 µl M-MulV reverse transcriptase, adjusted to 20 µl using DEPC-treated water. The cDNA synthesis was performed at 42°C for 1 h. Real-time RT-PCR was carried out.
using a Rotor-Gene 6000 (Corbet®). Each reaction mixture contained 2 μl of cDNA, 10 μl of SYBR Premix Ex Taq II (TaKaRa-Japan), and 1 μl of forward and reverse primers (5 μM), adjusted to 20 μl using dH2O. The expression pattern of the transcripts was analyzed using quantitative real-time RT-PCR. The list of primer sequences is shown in Table 2.

**Embryo transfer**

Bakhtiari recipient goats with at least one parturition and normal appearance and health were selected by an expert veterinarian and screened for contagious diseases, including Johnne's disease and brucellosis. The selected goats had a mean age of about 2-3 years and a mean weight of 35 kg. They were synchronized with the insertion of progesterone sponges, containing 40 mg fluorogestone acetate (Intervet™), and this was considered as day 0. Then, 500 IU of PMSG (pregnant mare serum gonadotropin), 250 μg of prostaglandin (estroPLAN®, Australia), and 1000 IU of hCG (human chorionic gonadotropin) were administered on days 5, 7, and 9, respectively. Sixteen days post-insertion of progesterone sponges, grade 1 and 2 blastocysts were selected from each group; then, two to four blastocysts were transferred to the synchronized goats using the laparoscopic embryo transfer technique. The establishment and progression of pregnancies in the recipient goats were measured using rectal ultrasound on days 28-38 and abdominal ultrasound on days 83-113 after embryo transfer, respectively. All the animals were allowed to undergo normal delivery [26].

**Statistical analysis**

Experimental data were presented as mean ± SEM. The differences between the data related to the two media were examined using independent sample t-test. The differences were considered statistically significant at P< 0.05.

**Results**

In vitro development of IVF, SCNT, and parthenogenetic embryos in two culture media (SOF vs. BO)

Cleavage rate was not significantly different between the experimental groups (P > 0.05) (Fig. 1). The blastocyst rate in the BO medium was higher than that of the SOF medium in the SCNT group (P < 0.05) (Fig. 1).

Effects of embryo culture medium on the genes expression of goat SCNT and IVF blastocyst

In the IVF group, OCT4, BMPR1, and GCN5 showed significantly higher expression in the SOF medium than in the BO medium. In this group, AKT, FGFR4, and SOX2 showed significantly lower expression in the SOF medium than in the BO medium (Fig. 2). In the SCNT group, FGFR4, GCN5, FZD, CTNNB, BMPR1, and FGFR4 showed significantly higher expression in the SOF medium than in the BO medium (Fig. 3). On the other hands, all of the genes expression were overexpressed in both of media, SOF and BO (Fig. 4, 5).

In-vivo development of SCNT goat embryos
The rate of pregnancy loss in the BO medium was lower than that in the SOF medium, but the difference was not significant (P>0.05) (Table 3). Full-term pregnancy and SCNT efficiency in the BO group were higher than those of the SOF group, but the difference was not statistically significant (P>0.05) (Table 3).

| group | Transferred Embryo | recipient | Established pregnancy % | Loss of pregnancy % | Full term pregnancy % | Live birth | SCNT efficiency % |
|-------|--------------------|----------|-------------------------|--------------------|-----------------------|-----------|-------------------|
| SOF1/SOF2 | 96 | 24 | 10(41.66 %) | 5(50%) | 5/10(50%) | 6 | 6/96(6.25%) |
| Bo | 100 | 25 | 11(44%) | 4(36.36%) | 7/11(63.63%) | 8 | 8/100(8%) |

Table 3
Retrospective and prospective results of in-vivo developmental competence of SCNT goat embryos developed in SOF1/SOF2 and BO culture media.

**Discussion**

More than a century has passed since the pioneering work of Wesley Kingston Whitten (1956), the father of embryo culture medium [36], and significant progress has been made in culturing human and, to some extent, domestic species zygotes to blastocysts and then to embryos [37]. However, recent studies on humans have shown that culture media composition and assisted reproductive technology (ART) procedures affect parturition [38], the weight of children at birth, and even their health after ART procedure [39]. Despite these achievements in culturing human embryos, there is a need for further research, especially in culturing embryos from domestic species towards complete epigenetic patterns. The results of our previous work [33] showed that despite producing a high blastocyst rate in the IVF- and SCNT-derived from caprine embryos, the commercial G1/G2 media, containing HSA-solution, used for culturing human embryos and tested by mouse embryonic assay had a negligible post-implantation developmental competence compared to that of the SOF medium, a homemade medium supplemented with BSA. This study was designed to compare the SOF medium with the BO medium using a single-step medium, containing synthetic serum [40] replacement, BSA, vitamins, amino acids, and antioxidants, for culturing parthenogenic, IVF-, and SCNT-derived caprine embryos. The results showed that although there was no significant difference between the groups in terms of the cleavage rate, the SCNT blastocyst rate was significantly higher in the BO medium compared to that of the SOF medium [36]. Based on the difference between the requirements of SCNT embryos and those of the parthenogenic and IVF-derived embryos, it was concluded that the BO medium was more advantageous for culturing SCNT embryos. The results regarding the post-implantation developmental competence of SCNT-derived embryos revealed that the two media had similar clinical pregnancy rates. However, the loss of pregnancy rate was lower in the BO medium compared to that of the SOF medium; consequently, the full-term pregnancy rate was higher in the BO medium, but the differences were not statistically significant.
In order to clarify whether this effect was related to the quality of blastocysts derived from these two media, the relative expression of sixteen genes in caprine embryos derived from the IVF and SCNT procedures cultured in the SOF and BO media were assessed during different developmental pathways, including pluripotency, FGF, TGFβ, cell cycle, proliferation, histone transferase, trophectoderm, and WNT.

There were no significant differences between the two types of embryos (IVF vs. SCNT) in terms of the gene expression of the cell cycle, proliferation, and trophectoderm signaling. Moreover, in the IVF-derived embryos, among the genes assessed, three genes, i.e. AKT, FGFR4, SOX2, which belonged to the FGF and pluripotency signaling pathways were down-regulated while another group of three genes, including OCT4, BMPR1, GCN5, which belonged to the pluripotency, TGFβ, and histone transferase signaling pathways were upregulated in the SOF medium compared to the BO medium. On the other hand, in the SCNT-derived embryos, the relative expressions of 5 genes, i.e. FGFR4, BMPR1, GCN5, CTNNB, FZD, out of the 16 genes were higher in the SOF medium compared to the BO medium.

According to the results of our previous work [41, 42], during the development of oocyte, zygote, and 8–16 cell embryos to day 7 blastocysts, of the 16 genes mentioned above, the relative expressions of 11 genes, including OCT4, SOX2, FGFR4, ERK1, AKT, BMPR1, SMAD5, CDC25, LIFR, CTNNB, and FZD, decreased as embryos reached the blastocyst stage and only the relative expression of one gene, i.e. CDX2, increased by day 7. These authors also used the same SOF composition. Therefore, those genes with reduced expression can be employed as positive selection markers to optimize the composition of the medium. The pregnancy rate for the transfer of IVF embryos cultured in SOF was 12.2% per embryos transfer and 39.28% per embryo transfer in the caprine embryos in our farm (data not shown). In this regard, it must be noted that among the several culture media used so far for SCNT and iSCNT embryo culture in domestic and wildlife species, the SOF medium has been the most commonly used medium across different species.

Despite some specific species differences, NANOG, SOX2, and OCT4 played key roles in the feedback loops in mammals. Therefore, any decrease in one of these factors can result in the up-regulation of one or two of this triad of factors [43]. In this study, OCT4 showed the highest expression relative to the reference genes. Therefore, the high expression of OCT4 in the IVF group in response to the decrease in the SOX2 expression was consistent with what has been reported in the literature, although no such difference was observed in the SCNT-derived embryos.

The segregation of cell lineages in early embryogenesis supports the establishment of pregnancy and the development of the fetus [44]. The FGF4 ligand during blastocyst formation may induce some responses in neighboring cellular compartments, and the emerging TE and ICM cells may establish a close relationship. The expression of some ligand receptors, including IL6/IL6R, FGF/FGFR, TGFβ/TGFBR, and BMP/BMPR, may play important roles in the coordinated development of the TE in preparation for implantation [45]. In mice, the FGF4 produced by the epiblast supported the expansion of the trophoblast stem cell niche. A similar cross-talk is also operating in the pig embryo, where FGF4 has a trophic effect during TE segregation and elongation, resulting in the formation of a one-meter-long trophoblast within a
few days. In day 3.5 embryo, the cells respond to FGF4, the expression of which is controlled by Oct4/Sox2 in ICM cells, to initiate cell differentiation into parietal endoderm (PE). The mutation in the FGF4 gene or its cognate receptors (FGFR1/2) or the chemical inhibition of FGF/MEK signaling can result in the inhibition of PE migration in the mouse embryo [44]. In summary, FGF ligand-mediated activation of FGFRs may promote a switch in the transcriptional profile of ICM from EPI- to hypoblast-associated gene expression [46, 47]. FGF4 appears to be the main mediator of this segregation in mouse embryos and the lack of it can result in the enrichment of NANOG. However, this effect in bovine embryos is not mediated through FGF, and in the goat embryos, it remains to be defined. In this study, the expression of FGFR4 in the IVF-derived embryos was higher in the BO medium compared to that of the SOF medium (Fig. 2). The opposite of this trend was observed in the SCNT-derived embryos, that is, the expression of FGFR4 was higher in the SOF medium compared to that of the BO medium. This difference highlights the difference between the two media used, the difference between the two procedures applied, and the need for a specific medium for each procedure.

The embryonic development and regulation of cell proliferation by Wnts depend on endogenous WNTs, receptors, signaling molecules, and the regulation of canonical and non-canonical pathways to fine-tuning the balance between pluripotency, self-renewal, and cell-fate commitment [48]. Similar to our previous observation [41, 42], the Wnt signaling in the IVF-SOF group was repressed in the goat blastocysts; this might reflect the poised state of developmental genes in the goat embryos compared to the bovine embryos. In this regard, the maintenance of pluripotency and the inhibition of blastomere differentiation caused a decrease in the rate of blastocyst formation [49]. The results showed that in the SCNT-SOF group, the expressions of CTNNB and FZD increased and the Wnt signaling was upregulated. Based on the above findings, we hypothesized that the endogenous WNTs, receptors, and signaling molecules may be different between the IVF and SCNT embryos. Moreover, it can be stated that Wnt regulation can possibly contribute to SCNT embryonic development.

**Conclusions:**

The only difference observed between the SOF and BO media was related to the embryo development-to-blastocyst rate, which was significantly higher in the BO medium compared to that of the SOF medium. However, no significant difference was observed between the two media in terms of post-implantation developmental competence. Based on these results, the higher expression of genes in the SOF medium compared to that of BO medium and the higher expression of genes in the SCNT vs. IVF embryos irrespective of the type of medium suggest that BO might be a better medium for *in vitro* culture (IVC) of caprine embryos. This finding is in agreement with the literature, which has shown that some of these genes are down-regulated during the blastocyst stage. In addition, these data suggest that to achieve a higher reprogramming efficiency in the SCNT procedure, further intervention is needed at different levels to optimize the application of SCNT technology in IVC media [32, 50, 51].

**List Of Abbreviations:**
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests:

The authors declare that they have no competing interests.

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Authors’ contributions:

MH Nasr-Esfahani, M Hajian and F Jafarpour perceived and designed the study; SM Aghamiri, M Rahimi and S Rouhollahi Varnosfaderani performed the experiments; MH Nasr-Esfahani, M Hajian and S
Rouhollahi Varnosfaderani wrote the manuscript. F Jafarpour and S Rouhollahi Varnosfaderani prepared the draft version of the article for submission. All authors read and approved the final manuscript.

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**Figures**
Figure 1

The in vitro development of IVF, parthenogenetic, and SCNT embryos in the BO and SOF media. The asterisks represent significant differences (P<0.05).

Figure 2

The genes expression of goat IVF blastocysts in the BO and SOF media. The asterisks represent significant differences (P<0.05).
Figure 3

The genes expression of goat SCNT blastocysts in the BO and SOF media. The asterisks represent significant differences (P<0.05).
Figure 4

The genes expression of goat SCNT and IVF blastocysts in the SOF medium. The asterisks represent significant differences (P<0.05).
Figure 5

The genes expression of goat SCNT and IVF blastocysts in the BO medium. The asterisks represent significant differences (P<0.05).

Supplementary Files

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