J Assist Reprod Genet (2008) 25:375–381
DOI 10.1007/s10815-008-9250-1

ASSISTED REPRODUCTION

# Clinical outcome of magnetic activated cell sorting of non-apoptotic spermatozoa before density gradient centrifugation for assisted reproduction

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Received: 12 March 2008 / Accepted: 3 September 2008 / Published online: 23 September 2008


## Abstract

*Purpose* Magnetic activated cell sorting (MACS) eliminates apoptotic spermatozoa based on the presence of externalized phosphatidylserine residues. We evaluated the outcome of male fertility treatment when intracytoplasmic sperm injection (ICSI) into human oocytes was performed with non-apoptotic MACS-selected spermatozoa.

*Methods* 196 couples were treated by ICSI following spermatozoa preparation by MACS (study group; 122 couples) or density gradient centrifugation (DGC) (control group; 74 couples). Fertilization, cleavage, pregnancy, and implantation rates were analyzed.

*Results* The percentage of sperm with normal morphology after MACS selection was improved. Cleavage and pregnancy rates were higher, respectively, in the study group than in control. A slightly higher implantation rate was also observed in the study group.

*Conclusions* MACS selection of human spermatozoa increased cleavage and pregnancy rates in oligoasthenozoospermic ART cases. This novel method for selecting non-apoptotic spermatozoa for ICSI is safe and reliable, and may improve the assisted reproduction outcome.

**Keywords** Apoptosis · Spermatozoa · Intracytoplasmic sperm injection · Pregnancy rate

*Capsule* MACS selection of human spermatozoa increased cleavage and pregnancy rates in oligoasthenozoospermic ART cases.

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## Introduction

Assisted reproductive technologies (ART) have become the treatment of choice in many cases of male and female infertility; however, the current success rates of these procedures remain suboptimal. One potential reason for these suboptimal rates may be the inclusion of apoptotic sperm during in vitro fertilization (IVF) [1, 2]. Sperm may have fragmented DNA despite a normal appearance and motility, and the use of such sperm in ART may have adverse effects on the outcome of the procedure [3].

Therefore, the quality of sperm samples is one of the factors that helps determine successful assisted reproduction [4]. A variety of sperm preparation techniques are available to select motile spermatozoa capable of fertilizing the female oocyte [5]. Successful fertilization requires a sperm plasma membrane with normal integrity and function [6]; however, impaired integrity of the spermatozoal membrane is frequent in infertile men, and contributes to infertility despite other normal sperm parameters [7, 8].

Apoptosis is a mode of programmed cellular death which leads the cell to suicide without eliciting an inflammatory response. Distinct markers are expressed by mature sperm cells in response to apoptosis-related cell damage [9]. In most normal, viable eukaryotic cells, the negatively-charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis. However, in necrosis, PS becomes accessible due to the disruption of membrane integrity. Additionally, PS also becomes accessible in activated platelets, in certain cell abnormalities like sickle cell anemia, in erythrocyte senescence, upon degranulation of mast cells, and in certain stages of B cell differentiation. PS exposure also serves as a trigger for the recognition and removal of apoptotic cells by macrophages [10].

Magnetic-activated cell sorting (MACS) is an excellent tool for separating cells of interest out of mixed cell populations. The sorter utilizes magnetic micro-/nano-particles conjugated with antibodies specific to the cell membrane protein of interest. The magnetic particle-bound cells lie in a high magnetic energy gradient and eventually change their pathway. Unbound cells are not influenced by the magnetic field and maintain their initial pathways [11].

Annexin V is a phospholipid-binding protein with a molecular weight of 35 kDa. It is a major cell membrane component of macrophages and other phagocytic cell types. Annexin V has a high affinity to PS in the presence of physiological concentrations of $Ca^{+2}$ and is unable to pass through intact sperm membranes [12]. Thus, annexin V binding by a sperm cell indicates that its membrane integrity has been compromised [13]. Annexin V can therefore be used to isolate cells with exposed PS by using annexin V MicroBeads [14].

Use of MACS allows the enrichment of cells either with or without PS exposed on their surfaces. Cells are magnetically labeled with annexin V MicroBeads and passed through a MACS column, which is placed in the magnetic field of a MACS separator. The magnetically-labeled, PS-exposing cells are retained in the column, while the unlabeled cells pass through. After removing the column from the magnetic field, the magnetically-retained PS-exposing cells can be eluted as the positively selected cell fraction. It was previously reported that mature sperm fractions show a lower incidence of apoptosis and higher nuclear maturity. The association of PS externalization with a high DNA stainability index in human semen samples indicates that early apoptosis is associated with immature nuclear development and abnormal chromatin [15].

It was previously reported that using MACS together with density gradient centrifugation (DGC) to prepare human spermatozoa was superior to other sperm preparation methods in terms of providing motile, viable and non-apoptotic spermatozoa. Apoptotic markers measured were levels of activated caspase - 3, integrity of mitochondrial membrane potential and externalization of phosphatidylserine residues and it was shown that combination of DGC and MACS was more effective than other techniques to reduce the percentage of apoptotic spermatozoa after preparation. [5, 14].

It is essential to isolate viable, motile sperm from semen with a concentration and motility appropriate for intra-cytoplasmic sperm injection (ICSI) treatment. Various methods of sperm isolation and preparation for ICSI have been developed, yet there are no randomized comparative studies evaluating the effectiveness of MACS separation on the outcome of ICSI cycles. Here, we aimed to compare the cleavage, fertilization, implantation, and pregnancy rates associated with two sperm preparation methods, MACS and DGC, for the ICSI of superovulated women.

## Materials and methods

This prospective study was designed to assess the impact of the MACS technique for selection of non-apoptotic spermatozoa on the outcome of ICSI, compared with DGC only. A total of 200 women who met our selection criteria were included in the study and were scheduled for ICSI treatment between October 2007 and January 2008. Inclusion criteria were primary infertility, maximum baseline FSH of 10 mIU/mL, maximum baseline $E_2$ of 75 pg/mL, ovulatory menstrual cycles, age at the time of screening of <35, no uterine abnormalities or communicating hydrosalpinx, and no history of low or absent ovarian response during FSH/HMG treatment. All of the men included in the study had oligozoospermia, asthenozoospermia, oligoasthenospermia (classified according to WHO criteria [16]), and/or teratozoospermia (classified according to Strict criteria [17]). Patients underwent ICSI treatment with cleavage stage (day 3) embryo transfer at the MAYA Center for Assisted Reproductive Technologies between January and March 2008 and were enrolled in the study. Frozen/thaw embryo transfer cycles and conventional IVF treatments were not included in the study.

Two hundred subjects meeting our inclusion criteria were scheduled for treatment and counseled to participate in either

the study or control group. All couples were enrolled in the treatment after informed written consent. One hundred and twenty four subjects agreed to participate in the study group and 76 subjects agreed to participate in the control group. Each participant had only one treatment cycle during the study period. A total of four cycles were cancelled before oocyte pick-up due to poor ovarian response; two from the study group and two from the control group. One hundred and ninety six couples completed the study; 122 subjects in the study group and 74 subjects in the control group.

Study group sperm cells were prepared by DGC after MACS of non-apoptotic spermatozoa. Control group sperm cells were prepared by DGS without magnetic sorting. Patient ages, duration of infertility, sperm count, motility, and morphology, the number of oocytes collected, the number of metaphase II oocytes injected, fertilization rates (total number of normal fertilized oocytes/total number of metaphase II oocytes injected), cleavage rates (total number of cleaved embryos on day 3/total number of normal fertilized oocytes), embryo quality (mean number of blastomeres per embryo on day 3 and mean percentage of fragmentation per embryo on day 3), number of embryos transferred, chemical and clinical pregnancy rates, and implantation rates (total number of viable sacs with fetal heartbeats/total number of embryos transferred) were compared among groups (Table 1).

For magnetic selection, spermatozoa were incubated with annexin V-conjugated microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) for 15 min at room temperature (RT). One hundred microliters of microbeads were used for each 10 million separated cells. The sperm/microbead suspension was loaded in a separation column containing iron globes, which was fitted in a magnet (MiniMACS; Miltenyi Biotec). The fraction composed of apoptotic spermatozoa was retained in the separation column, whereas the fraction with intact membranes was eluted through the column and was collected as non-apoptotic spermatozoa [14].

All sperm samples were prepared by SpermGrad single layer discontinuous DGC (Vitrolife, Kungsbacka, Sweden). Samples were loaded onto a 90% discontinuous gradient and centrifuged at 300 g for 20 min at RT. The pellet was washed twice by centrifugation for 10 min, and was resuspended in SpermRinse, bicarbonate buffered sperm wash media (Vitrolife).

The long protocol was preferred for superovulation in all of cases, based on the patient's age and the basal ultrasonographic evaluation of the ovaries. Recombinant FSH (rFSH, Gonal-F, Serono, Istanbul, Turkey) was applied s.c. for follicular growth, and 0.5 mg leuprolide acetate/day s.c. was used. Final oocyte maturation and ovulation stimulation were provided in all cases by 10,000 IU of HCG (Pregnyl, Organon, Istanbul, Turkey). After 35.5 h, oocytes were retrieved by transvaginal follicle aspiration under ultrasound guidance with total intravenous anesthesia.

Oocyte-cumulus complexes were identified with a dissecting microscope after oocyte retrieval. Before ICSI, cumulus and corona cells were removed enzymatically by 40 IU/mL hyaluronidase (Hyase, Vitrolife) and gentle pipetting. After denudation by hyaluronidase treatment, ICSI was performed using an Olympus Inverted Microscope IX 71 (Olympus Corporation, Shibuya-ku, Tokyo, Japan) equipped with a Narishige micromanipulator ON-2 (Setagaya-ku, Tokyo, Japan). The denuded oocytes were examined and only those that had extruded the first polar body (metaphase II) were microinjected. A single, motile, morphologically normal spermatozoon was immobilized with the injection pipette. Sperm was injected into the ooplasm at the 3 o'clock position, the polar body being oriented at the 12 o'clock position [18].

Injected oocytes were placed in fertilization medium (G-IVF, Vitrolife) for 16–18 h. After fertilization assessment, normally fertilized prezygotes were placed in a cleavage medium (G-1, Vitrolife) for 2 d. On day 3, embryos were evaluated and selected for embryo transfer. Embryos were transferred after a non-selective quarter laser-assisted hatching technique, using a solid state diode laser (Saturn 3 Laser System, Research Instruments, Cornwall, UK) in G-2 (Vitrolife).

**Table 1** Cycle characteristics of the study group and the control group

| | Study group | Control group | P-Value |
|---|---|---|---|
| N of cases | 122 | 74 | |
| Female age[a] | 28.96±4.55 | 28.35±4.77 | NS |
| Duration of infertility (years)[a] | 7.81±3.96 | 7.64±4.54 | NS |
| Sperm concentration ($\times 10^6$/mL)[a] | 10.793±13.55 | 9.63±17.79 | NS |
| Total sperm motility (%)[a] | 25,9±13,08 | 22.59±16.86 | NS |
| Progressive motility (%)[a] | 3.01±4.95 | 3.86±6.56 | NS |
| Total progressive motile sperm ($\times 10^6$)[a] | 1.39±4.71 | 1.29±3.43 | NS |
| Strict morphology (%)[a] | 0.918±0.949 | 0.919±1 | NS |
| Number of oocytes collected[a] | 14.65±7.88 | 15.69±9.53 | NS |
| Number of metaphase II oocytes injected[a] | 11.4±6.29 | 12.12±6.96 | NS |

[a] Values are mean plus or minus one standart deviation
NS = Not significant

Cycle characteristics and clinical outcomes were compared between two groups by analysis of variance, Fisher, and mid-p exact tests and Chi squares using OpenEpi version 2.2 software. This study was approved by the Local Ethical Committee of the Keçiören Education and Research Hospital, Ankara, Turkey.

## Results

Of the 196 patients that completed the study, 122 patients (study group) were treated by ICSI after MACS selection of non-apoptotic spermatozoa. The 74 control group members were treated by ICSI without magnetic selection (Table 1). Both groups had comparable demographic characteristics, with mean ages of females in the study and control groups of 28.96 and 28.35, respectively. An average of 14.65 and 15.69 oocytes was collected from each individual in study and control group, respectively. There were no statistically significant differences ($P$=NS) between the two groups in the duration of infertility, sperm concentration, total sperm motility, progressive motility, total progressive motile sperm count, strict morphology, or the number of metaphase II oocytes injected (Table 1).

The mean total sperm counts of patients in the study and control groups were $10.79\times10^6$/mL and $9.63\times10^6$/mL, respectively. The mean percentages of the total sperm motility of patients in study and control groups were 25.9% and 22.59%, respectively.

The mean percentage of sperm representing normal morphology in the study group was 0.918% vs. 0.919% in the control group (Table 1). In contrast, the percentage of sperm with normal morphology after MACS separation was 2.44% ($P$<0.01). Thus, the magnetic enrichment of non-apoptotic spermatozoa significantly improved the percentage of sperm with normal morphology according to Strict criteria (Table 2).

An average of 11.4 and 12.12 mature, metaphase II oocytes were retrieved and injected in the study and control groups, respectively. Normal fertilization with two visible pronuclei and a second polar body occurred at rates of 69.52% and 69.9%, respectively, in the study and control groups ($P$=NS) (Table 3). No fertilization failures were observed in either group.

**Table 2** Strict morphology results before and after MACS application

| | Raw semen | MACS seperation | P-Value |
|---|---|---|---|
| N of cases | 122 | 122 | |
| Strict morphology (Normal %)[a] | 0.918±0.949 | 2.443±1.97 | <0,01 |

[a] Values are mean plus or minus one standart deviation

**Table 3** Fertilization and cleavage rates and embryo quality in the study and control groups

| | Study group | Control group | P-Value |
|---|---|---|---|
| N of 2PN (Fertilization rate %)[a] | 7.93±4.88 (69.52%) | 8.47±5.21 (69.9%) | NS |
| N of embryos (Cleavage rate %)[a] | 7.7±4.52 (97,2%) | 7.47±4.11 (88.2%) | <0,01* |
| N of blastomeres per embryo[a] | 6.46±1.15 | 6.35±1.18 | NS |
| Fragmentation rate per embryo (%)[a] | 5.26±2.99 | 5.09±3.04 | NS |

[a] Values are mean plus or minus one standart deviation
* Odds Ratio 4.58 (2.93< OR >7.32) (Study group zygotes were more likely to cleave, Mid-P Exact)
NS = Not significant

Although there were no significant differences between the number of blastomeres per embryo on day 3 (6.46 vs. 6.35 in the study and control groups, respectively) or the fragmentation rates per embryo (5.26% vs. 5.09% in the study and control groups, respectively), there was a statistically significant improvement in the cleavage rates of the study group compared to the control group (97.2% vs. 88.2% in the study and control groups, respectively; $P$< 0.01, OR=4.58) (Table 3).

An average of 3.63 embryos were transferred into individuals of the study group vs. 3.5 embryos in the control group ($P$=NS) (Table 4). The number of chemical pregnancies to occur was significantly higher in study group ($P$<0.05, OR=1.87). The clinical pregnancy rate achieved after intracytoplasmic injection of MACS-selected non-apoptotic spermatozoa was 48.36% in the study group. Intracytoplasmic injection of spermatozoa after DGC without magnetic selection in the control group yielded a

**Table 4** Pregnancy and implantation rates among two groups

| | Study group | Control group | P-Value |
|---|---|---|---|
| N of cases | 122 | 74 | |
| N of chemical pregnancies (%) | 75 (61.47%) | 34 (45.95%) | <0,05** |
| N of clinical pregnancies (%) | 59 (48.36%) | 27 (36.49%) | 0,052* |
| N of embryos transferred[a] | 3.63±0.69 | 3.5±0.93 | NS |
| N of FHB (Implantation rate %) | 97 (21.9%) | 50 (19.31%) | NS |

[a] Values are mean plus or minus one standart deviation
** Odds Ratio 1.87 (1.04< OR >3.38) (Study group individuals were more likely to achieve chemical pregnancies, Mid-P Exact)
* Odds Ratio 1.63 (0.9< OR >2.96) (Study group individuals were more likely to achieve clinical pregnancies, Mid-P Exact)
NS = Not significant
FHB = Fetal heartbeat

clinical pregnancy rate of 36.49%. ($P$=0.052; OR=1.63) (Table 4). Although the implantation rates were statistically comparable between the two groups, a slightly higher rate was observed in the study group (21.9% vs. 19.31%, study vs. control groups) (Table 4).

## Discussion

Here we compared the cleavage, fertilization, implantation, and pregnancy rates associated with two methods of sperm preparation, MACS and DGC, for the ICSI of super-ovulated women. The percentage of sperm with normal morphology increased after MACS separation. Comparable numbers of oocytes were retrieved and injected in patients in the study and control groups, and no fertilization failures were observed in either group. Study group embryos showed significantly better cleavage rates than control group embryos and a higher chemical pregnancy rate was achieved after ICSI of MACS-selected non-apoptotic spermatozoa. A slightly **(**but insignificantly**)** higher clinical pregnancy and average implantation rate was also observed in the study group.

Apoptosis is an ongoing physiological phenomenon that maintains the number of germ cells within the supportive capacity of the Sertoli cells [19]. Although ejaculated spermatozoa display several apoptosis-like characteristics, these apoptosis-related features do not necessarily indicate death functions [20]. Nevertheless, the apoptosis-like phenotype of ejaculated sperm has been associated with the presence of abnormal spermatozoa in semen [21–23]. Failure to eliminate these abnormal spermatozoa during spermatogenesis, known as “abortive apoptosis,” may be the reason for their presence in semen [21, 23]. Activation of caspases, disruption of the mitochondrial membrane potential (MMP), and increased DNA fragmentation are some of the apoptotic features that have been identified in ejaculated spermatozoa [20, 24–26].

Another apoptotic event reported in human spermatozoa is the externalization of the PS, which is normally present on the inner leaflet of the sperm plasma membrane [27, 28]. Phosphatidylserine has a high and selective affinity for annexin V, a 35 kDa phospholipid-binding protein [29]; therefore, annexin V binding to a spermatozoon indicates that its cellular membrane is impaired. The binding process can be the result of translocation of PS from the inner to the outer leaflet of the plasma membrane, resulting in PS exposure on the external surface. This translocation is one of the earliest detectable features of cells undergoing the terminal steps of apoptosis [27].

Density gradient centrifugation reportedly isolates spermatozoa with higher MMP and DNA integrity [30, 31], but apoptotic spermatozoa cannot be eliminated by this technique. Magnetic cell sorting using annexin V-conjugated microbeads specifically targets spermatozoa with deteriorated membranes based on PS externalization to the outer membrane leaflet. In other words, MACS acts on sperm at the molecular level. Applying DGC together with MACS results in acceptable sperm yield cell loss, and this combination can be considered an effective sperm preparation technique to enhance sperm quality and function [5]. Our study demonstrates that higher cleavage and chemical pregnancy rates can be achieved by using the MACS technique for preparation of human spermatozoa together with DGC and our data shows a trend for improvement in terms of clinical pregnancy and implantation rates.

Annexin-negative sperm separated by MACS had a significantly lower percentage of DNA fragmentation compared to annexin-positive and control sperm. The percentage of spermatozoa showing chromatin decondensation following ICSI was significantly higher in annexin-negative compared to annexin-positive sperm, but was almost identical to that of the controls. No statistically significant correlations were detected between the percentage of DNA fragmented sperm and the percentage of spermatozoa showing chromatin decondensation following ICSI [32].

An association between apoptosis and DNA fragmentation was previously reported for human spermatozoa. However, apoptosis and sperm DNA fragmentation do not seem to affect the early stages of fertilization. Although MACS can be used to isolate spermatozoa with compromised genomic integrity, it does not increase the incidence of sperm chromatin decondensation following ICSI [24].

Idiopathic forms of male subfertility may be due to endocrine disruption due to environmental pollution [33, 34], reactive oxygen species (ROS), or genetic abnormalities [35]. Environmental factors are known causes of abnormal spermatozoa and ROS production. Furthermore, morphologically abnormal spermatozoa and seminal leukocytes are considered primary sources of ROS production in semen. Ejaculates with increased ROS production due to the presence of excessive leukocytes or immature or damaged spermatozoa should not be separated by means of conventional swim-up or one-step washes. In these techniques, all the ejaculate cellular content is forced to the bottom pellet, allowing leukocytes and abnormal sperm to encounter and damage mature spermatozoa. In contrast, DGC has a great potential in the isolation of mature, leukocyte-free spermatozoa, and MACS is a simple and fast sorting system for the separation of large numbers of cells. The leukocytes and morphologically abnormal spermatozoa can be isolated and excluded from sperm suspensions by means of MACS using paramagnetic microbeads [36]. Removal of abnormal spermatozoa decreases the oxidative stress among vital and healthy cells. Thus, another potential reason for the higher cleavage rates achieved after MACS

separation in this study might be related to decreased oxidative stress in the ejaculate after magnetic selection. On the other hand, further studies are needed to show the influence of magnetic colon passage on reduction of oxidative stress without annexin V incubation.

As PS externalization and DNA fragmentation are higher in the ejaculated spermatozoa of translocation carriers, and as concentration and forward motility are higher in normal donor sperm, this suggests a biological process excluding unbalanced gametes. Translocation carriers are known to have higher rates of apoptosis compared to normal donors [37], and genetic abnormalities may result from these elevated apoptosis levels. Our study presents a higher cleavage rate and our data represent a trend for the improvement of clinical pregnancy and implantation rates, which may be correlated with the elimination of genetically abnormal spermatozoa due to their apoptotic features. Thus, MACS selection of human spermatozoa may result in the elimination of unbalanced gametes and in better pregnancy rates. Further studies should be conducted to better characterize this process.

Alterations in MMP or in PS externalization are characteristics of early stages of apoptosis in somatic cells. These alterations precede other manifestations of programmed cell death, such as DNA fragmentation. Previous studies have demonstrated a significant positive correlation between PS externalization and the loss of MMP. Although mitochondrial alterations have been associated with sperm disorders in infertile men regardless of apoptosis, and although PS externalization can be related to other sperm conditions, such as capacitation, the observed strong correlation provides further evidence that sperm in the early stages of apoptosis may indeed be present in the ejaculated spermatozoa. Further studies are needed to determine the mechanisms leading to plasma and mitochondrial membrane alterations in human sperm [38].

The poor sperm morphology profile seen in the apoptotic sperm fractions may be partly due to the isolation of sperm with acrosomal damage and/or cytoplasmic droplets. It may also be partly due to technique-induced damage to the sperm midpiece or tail [39]. Sperm morphology is one of many factors affecting the outcome of ART. As apoptotic fractions represent highly abnormal spermatozoa, it should be expected that elimination of these cells would result in better pregnancy rates. Our study shows that higher pregnancy rates are obtained after the removal of apoptotic spermatozoa by MACS. It was proposed that sperm deformity index (SDI) scores were significantly reduced following DGC compared to the raw semen samples. Significantly lower SDI scores were also detected in a MACS/DGS-prepared annexin-negative fraction compared to spermatozoa prepared by DGC only [40]. In our study, we obtained significantly higher normal sperm morphology rates after MACS separation. The significantly higher cleavage rates and although not significantly, higher pregnancy and implantation rates achieved by ICSI of MACS-selected spermatozoa can be explained by better elimination of abnormal spermatozoa.

## Conclusion

The selection of human spermatozoa by MACS before DGC improved the cleavage, chemical and clinic pregnancy rates of oligoasthenozoospermic patients receiving ART. Since previous studies reported that using MACS together with DGC to prepare human spermatozoa for ART was superior to all other sperm preparation methods in terms of providing motile, viable and non-apoptotic spermatozoa [5, 14], it is acceptable to conclude that observed elevation in cleavage and pregnancy rates in the current study should be related to better elimination of apoptotic spermatozoa after MACS technique. But, since there might be some reasons for PS exposure other than apoptosis [27], advanced techniques are needed for confirming these results.

In conclusion, the selection of human spermatozoa by MACS is a safe and reliable method to prepare sperm cells for ICSI. Further studies should be carried out to clarify the underlying features of MACS-selected spermatozoa in order to interpret the higher cleavage and pregnancy rates. Nevertheless, the value of integrating MACS in sperm preparation prior to ICSI would be statelier after carrying out prospective randomized studies. In addition, certain subgroups of male infertility should be evaluated independently.

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