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# Isolation and Culture of Oocytes from Dairy Cows' Ovaries for Productivity in the Southeastern Region of Kazakhstan

**[1]Dauren Maratovich Bekenov, [2]Batyrkhan Azimkhanovich Buralkhiev, [3]Yusupzhan Artykovich Yuldashbayev, [1]Dastanbek Asylbekovich Baimukanov, [1]Makpal Temirkhanovna Kargayeva and [2]Gulzat Gabitkyzy Gabit**

*[1]Department of Animal Husbandry, Veterinary Medicine and Feed and Milk Quality Assessment, Scientific and Production Center for Animal Husbandry and Veterinary LLP, Astana, Kazakhstan*
*[2]Department of Animal Engineering, Kazakh National Agrarian Research University, Almaty, Kazakhstan*
*[3]Department of Private Animal Science, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy, Moscow, Russia*

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Corresponding Author:
Makpal Temirkhanovna Kargayeva
Department of Animal Husbandry, Veterinary Medicine and Feed and Milk Quality Assessment, Scientific and Production Center for Animal Husbandry and Veterinary LLP, Astana, Kazakhstan
Email: kargaevamakpal38@gmail.com

**Abstract:** This research work aims to study methods for isolating and culturing follicular oocytes from Holstein cows *in vitro*. The proven complex of technologies for embryo transplantation on an industrial scale will provide a 3-5-fold acceleration of reproduction of highly productive cows. As a result of scientific research, biotechnological methods of accelerated reproduction of dairy cattle have been substantiated and introduced into production. The object of the study was eggs obtained from the ovarian follicles of cows and heifers of breeding age of the Holstein breed, forced (poisoning, severe injuries, fractures, burns) slaughtered at the slaughterhouse of the Bayserke agro holding (the largest agricultural agro forming with an innovative approach in agriculture, a cluster uniting agricultural scientists and production workers. The main activities of the organization are animal husbandry, crop production, deep processing of products, greenhouse farming, and beekeeping, there is a dairy plant and a meat processing plant, at the launch stage there is a fish farm and a feed mill, scientific research and education) in Almaty region. When obtaining the material, the authors used ovaries without visible signs of pathology, at the stage of follicular growth, or with a developed corpus luteum. Based on the results of the research work, the quantitative yield of morphologically complete follicular oocytes in the amount of 1243, obtained from the ovaries of slaughtered cattle uteruses was established, depending on the season of the year, and their morphological state was also characterized. The nature of the influence of various cultural systems on the degree of maturation of follicular oocytes *in vitro* was also clarified, where 356 oocytes were cultured in the Dulbecco medium with 10% homologous serum, in the follicular fluid and in the IVF Universal medium 428 and 459 oocytes, respectively and a positive effect on the maturation of oocytes of the synthetic culture medium IVF Universal was established. The experiments and the methodology used for conducting research on laboratory animals comply with the requirements of biological safety and ethical principles of animal experimentation set out in the European Convention for the protection of vertebrates used for experimental and other scientific purposes.

**Keywords:** Oocyte, *In vitro* Fertilization, *In vitro* Method, Ovary, Egg, Follicle, Cultural Environment

## Introduction

The dairy production sector, as well as the beef meat production sector, underwent a significant decline after the collapse of the Soviet Union. The decrease in milk production that has occurred in the country is a consequence not only of a decrease in the number of dairy cows but mainly as a result of the loss of production efficiency of the remaining cows. This drop in production efficiency also had its origins as a result of the loss of the genetic potential of dairy cows. The loss of the genetic potential of cattle has had a negative impact on the production of dairy products, since in the case of dairy cows, the impact is directly on the amount of milk that cows can produce.

In this regard, within the framework of this study, it is planned to reproduce high-breed dairy cattle in a short time by applying biotechnological methods.

A promising method for increasing the genetic role of female cows today is the method of *in vitro* fertilization and *in vitro* culturing of oocytes, which allows a sharp increase in the number of their descendants. The effectiveness of this method is that eggs can be obtained from females all year round and over the lifetime of the animal (Makita *et al*., 2016; Hatırnaz *et al*., 2018; Ferré *et al*., 2020). The issues of optimizing the processes of *in vitro* fertilization of oocytes and the development of embryos in culture are of great practical importance in connection with the increasing use of the *in vitro* method of fertilization of oocytes. In theoretical terms, the study of these issues is necessary for the development of ideas about molecular cellular factors regulating reproductive function (Makita *et al*., 2016), as well as for the successful implementation of biotechnological programs for obtaining clones and transgenic animals (Yum *et al*., 2018). Given that the internal quality of the oocyte is one of the main factors influencing blastocyst yield, the precise identification of non-invasive cellular or molecular markers that predict oocyte competence is of great interest for research and practical work (Aguila *et al*., 2020). Under natural conditions, in cows and heifers of breeding age, a maximum of 5-7 oocytes are realized per 75-275 thousand oocytes, since the reproductive potential of females is hampered by the need to bear a fetus. This obstacle can be eliminated by using methods for obtaining a large number of oocytes from one female. It can be carried out by puncturing the follicles during laparoscopy (Ovum pick-up technology) or when slaughtering an animal directly from the ovaries. This method includes the isolation of eggs from the ovaries, their maturation in nutrient media, and fertilization outside the body, followed by transplantation to recipients (Saleem *et al*., 2022).

Many factors influence successful *in vitro* fertilization of oocytes. The main one is the correct selection of oocytes and the choice of culture media used for cultivation. As you know, gametes located in follicles are at the stage of meiotic prophase and are not ready for sperm penetration and fertilization. Moreover, meiosis in each sexual cycle is activated only in a very limited number of cells, which not only have to continue dividing but also have the opportunity to complete all stages of maturation in the follicle. As a result, it is far from indifferent at what stage the egg will be extracted from the ovary of cattle: It may turn out to be morphologically and functionally not ready for *in vitro* fertilization. The solution to this obstacle is the development of methods for culturing and maturing eggs "*in vitro*". It is necessary to extract an egg from the ovary of cows or heifers of breeding age, even if it has not completed all stages of maturation, and provide it with the opportunity in a culture medium, in an incubator, to complete all stages of development natural for subsequent fertilization (Shakura *et al*., 2018).

To date, two methods have been developed for culturing immature eggs: Cultivation in synthetic media and follicles. As a biologically active factor, blood serum is used, containing components that promote the survival and development of cells, including Insulin-like Growth Factor (IGF) and Epidermis Growth Factors (EGF), Platelets (PTF), and Fibroblasts (IGF). In addition to blood serum, normal maturation is ensured by the introduction of pituitary and sex hormones into the culture medium (Fortune *et al*., 2011).

It was previously found that almost 95% of oocytes extracted from antral follicles are at the diplotene stage and the rest are at the later stages of diagenesis metaphase I and II (Michalczyk *et al*., 2021).

Oocytes recovered from follicles resume meiosis spontaneously and can undergo all stages of maturation until metaphase II without any hormonal influences. However, this process is not equivalent to the maturation that occurs in the ovarian follicles before ovulation. Under cultivation conditions, only maturation of the nucleus occurs without the participation of the cytoplasm. As a result, the oocyte may not develop further after fertilization, since cytoplasmic factors play a major role in fertilization (Lunenfeld *et al*., 2019).

In many mammalian species, during *in vitro* fertilization and cultivation of early embryos, a block of development occurs at various stages. Thus, when culturing mouse embryos, embryogenesis is blocked at the 2-cell stage (Wang *et al*., 2021). In cows, the embryonic block is formed at the stage of 8-16 cells. However, this block is reversible, i.e. development can continue under natural developmental conditions, that is, after transplantation into recipients (Asgari *et al*., 2012).

As has been established, according to various studies, the proportion of oocytes matured to metaphase II in synthetic culture media varies widely. Therefore, researchers are faced with the task of improving cultural

systems. For these purposes, an urgent task is to search for modifications that most fully realize the potential of follicular oocytes, to reproduce natural development conditions outside the body (Michalczyk *et al*., 2021).

### *Aim of the Study*

The purpose of this study is the isolation and cultivation of follicular oocytes of Holstein cows in the conditions of the dairy complex of the agricultural holding "Baiserke-Agro".

Tasks:

1. To establish the quantitative yield of morphologically complete follicular oocytes of cows obtained from the ovaries of slaughtered animals, depending on the season of the year and their morphological condition is characterized
2. To find out the nature of the influence of various cultural systems on the degree of maturation of follicular oocytes *in vitro*, a positive effect on the maturation of oocytes of the IVF Universal synthetic culture medium has been established

## Materials and Methods

Isolation of oocytes: Obtaining eggs from the follicles of slaughtered animals is the simplest and most effective method.

The novelty of this study lies in the use of this method in the dairy complex of South-Eastern Kazakhstan for accelerated reproduction of dairy cattle with high genetic potential.

For our experiments, we used eggs obtained from the ovarian follicles of Holstein cattle after their slaughter. Ovariectomized cow ovaries were placed in a 20 mL tube with Phosphate Buffered Saline (PBS) medium, (a balanced saline solution used for various applications in cell culture, including washing cells before dissociation, transporting cells or tissues, diluting cells for counting and preparing reagents. DPBS does not include calcium and magnesium), in an amount of 10-13 pieces and delivered to the laboratory.

In laboratory conditions, the ovaries were washed 2-3 times in a warm saline solution with antibiotics in a ratio of 50 μg/mL streptomycin and 100 units/mL penicillin to remove external contaminants. The washed ovaries were transferred one at a time into a 40 mm Petri dish, into which 1.5-2 mL of 199 medium with Hanks salts was poured, which was previously placed in an incubator for 10-12 h to saturate with carbon dioxide (Wu and Zan, 2012).

The ovarian follicles were then cut under a laminar flow hood with a scalpel so that the follicular fluid was directly infused into the medium. After aspiration of the follicles, the ovary was removed and the dish with the medium was examined under a microscope. It should be noted that during aspiration of ovarian follicles, oocyte-cumulus complexes enter the environment along with the follicular fluid. The oocyte-cumulus complexes are the part of the follicular epithelium that is in direct contact with the oocyte during follicular development. In addition, the follicular fluid contains fibrinogen, which forms clots, and already 2-3 min after aspiration, oocytes are difficult to detect under a stereomicroscope and to wash off various contaminants. To solve this problem, two methods were used: (a) The follicular fluid was examined under a stereomicroscope immediately after aspiration (obtaining oocytes from mature follicles) and the found oocytes were immediately placed in a clean medium; (b) Follicular fluid was collected in dishes containing heparin medium, which prevented gelation of the medium.

But in the first option, it is not always possible to find oocytes in time and therefore the second option was also used. For this purpose, heparin was used at a concentration of 50 IU/mL. 1 mL solution was added to an 8 and 10 mL tube, giving a final concentration of heparin in the follicular fluid of 5 and 8 IU/mL (Hamano *et al*., 2021). When eggs were detected, they were collected using a pipette with a glass capillary and transferred to a separate four-well dish with oocyte culture medium, which was also previously in the incubator for 10-12 h. After the procedure, the number of oocytes was counted and to wash from follicular cells and tissues were retransferred to their clean well with the medium.

Cultivation of oocytes: Before cultivation, a quick morphological assessment of oocytes was performed; eggs with damaged zona pellucida were rejected. Oocytes of a round shape with homogeneous cytoplasm, a uniformly wide zona pellucida, and surrounded by multilayered compact follicular cells were considered suitable for research Skotti (2008).

Cultivation for oocyte maturation was carried out in an incubator with 5% $CO_2$ in air at a temperature of 37±0.1°C and a humidity of 85%. These conditions are met when using $CO_2$ incubators that maintain the required temperature, humidity, and $CO_2$ level in the chamber, which is maintained by supplying carbon dioxide from a cylinder, where it is in a liquefied state, and air from the environment. The humidity in the chamber of $CO_2$ incubators is maintained by the constant evaporation of bidistilled water from the bottom of the chamber from a special tray (Kassens *et al*., 2015). The pH level of the environment is 7.4, which corresponds to the pH of the blood; this acidity is optimal for fertilization and preimplantation development of oocytes and embryos (Gatimel *et al*., 2020).

The eggs were cultured in 1 mL four-well dishes in 800 μL of egg culture medium. Oocytes were transferred into separate wells of a four-well dish at the rate of 12-15 pieces per well. The oocytes, cultured for 24 h in an incubator at a temperature of 37ºC with 5% $CO_2$, were

transferred with a pipette into a clean medium for culturing oocytes, which had previously been in a $CO_2$ incubator for 12 h.

## Results and Discussion

In our experiments, to obtain eggs, we used the ovaries of mature animals after their slaughter, from which oocytes were isolated by aspiration of follicles. As a result, during the experiment, 96 ovaries were obtained from 48 slaughtered mature cattle. To extract oocytes, a total of 93 ovaries of reproductive cattle without obstetric-gynecological pathology at the stage of follicular growth or with a developed corpus luteum were used (Table 1).

During the period of experimental work, a total of 1516 oocytes were obtained from the ovaries of 48 cows. The table shows that during a morphological assessment under a stereomicroscope, 1243 oocytes, or 82% of the total number of eggs were selected for further maturation and 18% were discarded due to damage to the integrity of the zona pellucida and the state of the cytoplasm. Of the degenerate 67% or 183, micro traumatic damage occurred during the extraction of follicles, loss of transparency, and with a thickened transparent membrane, in 33% of the eggs the cytoplasm was fragmented and the presence of vacuoles was observed and there were also a dark color and the cytoplasm was wrinkled.

As a result, the average yield of eggs per ovary of animals was 16.3±0.42, of which, during morphological assessment of usefulness, an average of 13.37±0.36 was selected for further cultivation and fertilization under a stereomicroscope. The variability in the yield of the total number and morphologically completed oocytes is 24.9 and 26.4%, respectively. The number of degenerated ones averaged 2.93±0.16 with fluctuations of 55.7% (Table 2). The maximum and minimum values of oocytes isolated from one ovary ranged from 6- 32.

The influence of the seasons on the effectiveness of obtaining cow oocytes *in vitro* was also noted. It has been established that the autumn months are the most favorable for oocyte release. Apparently, the results are affected by seasonal changes associated with metabolic processes and changes in the hormonal balance in the body of cows.

On average, the yield of oocytes most suitable for obtaining complete embryos in the autumn months (September-November) was 17.3±0.68, of which 13.9±0.63 were morphologically completed. In the spring months (March-May) this figure was 16.34±0.74 and 13.69±0.66, respectively. In the summer (June-July), these indicators decreased slightly, amounted to 15.26±0.72, and morphologically completed 12.58±0.60, while the number of degenerated ones was in the range of 2.65-3.27 with a coefficient of variation of 54.8-60.2%. A fairly high yield of oocytes in the autumn months indicates, perhaps, that the body is preparing for the breeding period and therefore a larger number of follicles in the preovulatory phases are observed in the ovary.

Thus, the difference in the yield of the most suitable morphologically completed eggs in the autumn and summer seasons was 2.04 (Table 3).

**Table 1:** Morphological characteristics of oocytes

| Ovules | Total number, n | % |
|---|---|---|
| Quantity, total | 1516 | 100 |
| Morphologically qualitative | 1243 | 82 |
| Degenerate | 273 | 18 |
| Of them: | | |
| With shell damage | 183 | 67 |
| According to the state of the cytoplasm | 90 | 33 |

**Table 2:** Morphological characteristics of oocytes

| Indicators | M ± m | б | With v % |
|---|---|---|---|
| Total quantity | 16.30±0.42 | 4.056 | 24.9 |
| Morph. completed | 13.37±0.30 | 3.520 | 26.4 |
| Degenerated | 2.93±0.16 | 1.630 | 55.7 |

**Table 3:** Average yield of follicular oocytes depending on the season of the year

| Indicators | | Number of ovaries | n, oocytes | M ± m | б | Cv % |
|---|---|---|---|---|---|---|
| Autumn period | Total | 33 | 572 | 17.30±0.68 | 3.940 | 22.7 |
| | Morph. completed | - | 459 | 13.90±0.63 | 3.620 | 26.1 |
| | Degenerated | - | 108 | 3.27±0.32 | 1.840 | 56.3 |
| Spring period | Total | 26 | 425 | 16.34±0.74 | 3.780 | 23.1 |
| | Morph. completed | - | 356 | 13.69±0.66 | 3.360 | 24.6 |
| | Degenerated | - | 69 | 2.65±0.31 | 1.590 | 60.2 |
| Summer period | Total | 34 | 519 | 15.26±0.72 | 4.231 | 27.7 |
| | Morph. completed | - | 428 | 12.58±0.60 | 3.550 | 28.2 |
| | Degenerated | - | 96 | 2.82±0.26 | 1.540 | 54.8 |

We used several variants of cultural systems developed based on Dulbecco's medium and follicular fluid, which, according to the authors, allows us to achieve high and sustainable development outside the body, as well as a ready-made solution IVF-Universal (MediCult) intended for fertilization of oocytes and cultivation of embryos "*in vitro*". The cultivation time was determined based on the degree of development of follicular oocytes, which was assessed by morphological assessment. It has been determined that about 75% of oocytes isolated from ovarian follicles are at the fourth stage of the first prophase of meiosis, during which a crossover is formed between the paired chromatids of homologous chromosomes and then they begin to separate and a meiotic block occurs, controlled by the components of the follicular fluid and during isolation and cultivation "*in vitro*" can resume meiosis and go through all stages of maturation until metaphase II, the rest are at later stages of development.

To study the nature of the influence of various cultural systems on the degree of oocyte maturation, the experiment was carried out in three stages using various cultural systems (Dulbecco's medium with 10% homologous serum, 100% follicular fluid, IVF-Universal).

### *Results of Oocyte Cultivation in Various Cultural Media*

As a result, 1243 morphologically completed oocytes isolated from animal ovarian follicles were divided into three groups based on the composition of the nutrient media for ripening.

356 oocytes were cultured in Dulbecco's medium with 10% homologous serum; 428 and 459 oocytes were cultured in follicular fluid and IVF-Universal medium, respectively. In this case, the oocytes were divided according to three characteristics: Those with characteristic morphological signs of maturation, those without signs of maturation, i.e., unchanged and degenerated, that is, with wrinkled or fragmented cytoplasm. As mentioned above, for cultivation and fertilization, we selected oocytes with a diameter of at least 140-150 microns, that is, those closest to the size of a mature egg, as well as those surrounded by a mass of follicular cells well adjacent to the oocyte membrane (Table 4).

As can be seen from the data in Table 4, the largest percentage of oocytes with signs of maturation were characterized when cultivating eggs in IVF-Universal medium, which amounted to 68% or 312 of the total number. At the same time, the number of degenerated oocytes is relatively low and amounts to only 8%. Characteristic morphological signs of maturation of oocytes cultured in Dulbecco and VF media were observed in 18 and 22.9%, respectively. The percentage of degenerates in Dulbecco's medium was much higher than in other media (11.2 and 8% in IVF) and amounted to 23% or 82 oocytes out of 356 (Fig. 1).

The fully mature egg of cows and heifers of breeding age was characterized by a uniformly surrounded zona pellucida, more than one layer of cells of the corona radiata, and a large number of cells of the oviductal tubercle. At this moment in the egg, as a rule, the first meiotic division has already been completed, as a result of which the first polar body has separated and the second meiotic division is at the metaphase stage. At this stage, meiosis stops in the egg, which resumes only upon fertilization, that is, upon penetration of the sperm of a pre-selected frozen-thawed seed of a sire of a given breed for the oocyte donor.

Assessment of the degree of oocyte maturity based on the state of QAA is, as a rule, subjective and often does not correspond to the true state of the oocyte and therefore the final result of oocyte maturation "*in vitro*" is determined after their fertilization, the formation of pronuclei and further development of the embryo.

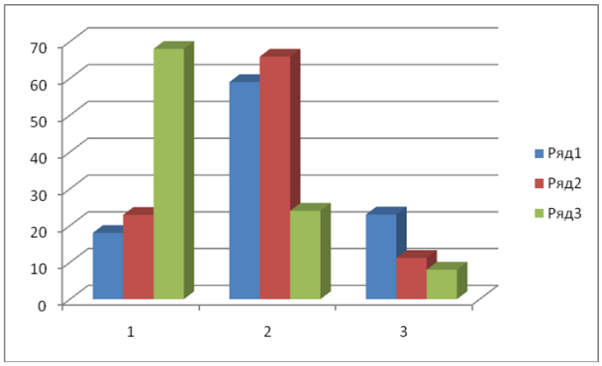


**Fig. 1:** Degree of oocyte maturation in culture media; Note: (Blue Dulbecco, red VF, green IVF Universal); 1st column number of mature oocytes; 2nd column no signs of maturation; 3rd column number of degenerated

**Table 4:** Results of follicular oocyte cultivation

| | Dulbecco with 10% homo. Serum follicular fluid | | IVF – Universal | | | |
|---|---|---|---|---|---|---|
| Medium name | n | % | n | % | n | % |
| Number of oocytes | 356 | 100 | 428 | 100.0 | 459 | 100 |
| With characteristic signs of maturation | 64 | 18 | 98 | 22.9 | 312 | 68 |
| No signs of maturation | 210 | 59 | 282 | 65.9 | 110 | 24 |
| Degenerated | 82 | 23 | 48 | 11.2 | 37 | 8 |

In countries such as the USA, and Canada, as well as in Western European countries with developed animal husbandry, almost more than 70% of breeding bulls arrive at artificial insemination stations with the brand "T" transplant. It should be noted that Israel ranks first in the world in terms of the dairy productivity of its herd. Highly effective scientific developments on embryo transplantation carried out by Maxximilk company make it possible to dramatically increase dairy productivity and profitability of the farm in record time - to raise a new elite herd of dairy cows in 32 months.

As already noted, *in vitro* fertilization and cultivation of mammalian oocytes "*in vitro*" is a necessary link in the accelerated reproduction of valuable genotypes of farm animals, as well as in conducting experimental embryological studies, embryo technological work on cloning, producing transgenic animals, isolating the intracellular mass of blastocysts, obtaining monozygotic embryos and in many other areas of modern biotechnology, namely in cell engineering. During extracorporeal cultivation of cow follicular oocytes in various cultural systems, in a comparative aspect, it was found that the highest percentage of maturation is observed in the synthetic IVF-Universal medium, 64% versus 16.8% and 21.8%. It was also found that the seasons have a slight influence on the efficiency of obtaining follicular oocytes from the ovaries of slaughtered animals. In the spring months, this indicator was 16.34±0.74, in the summer 15.26±0.72, and 17.3±0.68 in the autumn months.

*Summary*

It should be noted that in the ovaries of sheep in the autumn months, there are a greater number of preovulatory follicles than in comparison with the summer period, which possibly explains these indicators associated with metabolic processes, changes in the hormonal balance in the body of cows and with the preparation of the body for the breeding period. After all, as is known from the biological characteristics of cows, the seasonal manifestation of estrus occurs in the autumn months - from early October to mid-December. The total number of isolated follicular oocytes averaged 16.3±0.42, with a coefficient of variation of 24.9%.

The use of the *in vitro* method of embryos of a known sex for animal breeding can significantly increase the profitability of dairy and beef cattle breeding (Nikitkina *et al*., 2011).

## Conclusion

1. The quantitative yield of morphologically completed follicular oocytes of sheep obtained from the ovaries of slaughtered animals was established depending on the season of the year and their morphological state was also characterized
2. The nature of the influence of various cultural systems on the degree of maturation of follicular oocytes *in vitro* was clarified and the positive effect of the synthetic culture medium IVF-Universal on the maturation of oocytes was established

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## Author's Contributions

**Dauren Maratovich Bekenov:** Principal investigator, responsible for the experimental component of the research. Corresponding author, manuscript preparation. Contributed significantly to the implementation and preparation of the article.

**Batyrkhan Azimkhanovich Buralkhiev:** Contributed significantly to the implementation and preparation of the article.

**Yusupzhan Artykovich Yuldashbayev:** Conducted experimental data analysis. Contributed significantly to the implementation and preparation of the article.

**Dastanbek Asylbekovich Baimukanov:** Originator of the research idea, responsible for data analysis and synthesis. Contributed significantly to the implementation and preparation of the article.

**Makpal Temirkhanovna Kargaeva:** Conducted experimental data analysis. Contributed significantly to the implementation and preparation of the article.

**Gulzat Gabitkyzy Gabit:** Executor, responsible for analyzing research results. Contributed significantly to the implementation and preparation of the article.

## Ethics

The authors declare that there are no conflicts of interest with any third-party organizations.

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