

## EXPERIMENTAL TRIES TO ESTABLISH THE PREIMPLANTATIONAL MAMMALIAN EMBRYOS VIABILITY THROUGHOUT STAINING

### ÎNCERCĂRI DE STABILIRE A VIABILITĂȚII EMBRIONILOR PREIMPLANTAȚIONALI DE MAMIFERE PRIN TESTE DE COLORARE

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*Presently there are more methods to assess embryo quality but, still the widely used remains the morphological criteria method. In this experiment were tested two staining methods for embryos and oocytes. The embryos were recovered from mouse female at 72 hours after mating. The recovered embryos were first evaluated after morphological criteria and then by Trypan blue exclusion and Neutral red staining. Using Trypan blue exclusion were evaluated 30 embryos from which 19 (63.3) were classified as viable and 11 (36.7) were classified as nonviable. By Neutral red staining were evaluated 37 embryos from which 24 (64.8) were considered viable and 13 (35.2) were considered nonviable. The oocytes recovered were also evaluated using the two methods: using Trypan blue exclusion were stained 10 oocytes from which 9 remained uncolored and were considered viable and 1 was stained in blue and was considered nonviable and using Neutral red 13 oocytes were stained from which 9 were evaluated as viable and 4 as nonviable.*

**Key words:** embryos viability, Trypan blue exclusion, Neutral red staining

#### Introduction

One of the most important steps of embryo transfer procedure is represented by embryo quality evaluation.

The accurate evaluation of embryos suitable for transfer on receptor females influence the pregnancy rates and at the same time the embryo transfer efficiency.

The assessment of embryos can be made using different methods. These methods must be simple, accurate and non-invasive for the embryos, must offer the results in a very short period of time, should not require high preparation skills and have to be suitable for embryo evaluation directly in farms.

The vital staining dyes test the embryos viability either by detecting some modifications in cellular membrane permeability either/or inducing certain physiological states by exclusion or inclusion and retention of some dyes.

## Material and Methods

To assess the viability of mouse embryos and oocytes for this study were used 2 staining methods: Trypan blue exclusion and Neutral red staining. The embryos were recovered at 72 hours from vaginal plug, from mouse females without superovulation. The oocytes were recovered at 12 hours from the oestrus.

Trypan blue exclusion is a staining method which allows differentiating cells based on the capacity of viable cells to exclude this dye. For mouse embryos and oocytes staining was used a 0.4% Trypan blue working solution. Before staining the solution was incubated for 2 hours at 37°C. The embryos were placed on a Petri dish in 300µl 0.4% Trypan blue and then incubated at 37°C for 15 minutes. The embryos were washed then couple of times in PBS medium and microscopic evaluated. The embryos colored in blue were considered non viable and the uncolored were considered viable.

Neutral red is a dye widely used in different staining protocols to evaluate cells viability. For embryos and oocytes assessment was used 2% Neutral red solution. The working solution was prepared before use from a Neutral red stock solution (5 mg Neutral red dissolved in 1 ml ethanol, incubated at 37°C for 2 hour prior staining). The working solution was once more diluted 1:1 with preheated medium M2 before staining. For staining, the embryos were placed on a Petri dish in 300µl Neutral red solution prepared as above and then incubated at 37°C for 10 minutes. After this period, the embryos were washed couple of times in M2 medium and then were microscopic evaluated. The embryos and oocytes colored in red were considered viable (in these embryos existed enzymatic activity at the staining moment) and the uncolored were considered non viable.

## Results and Discussions

From 10 females used for this experiment were obtained 67 embryos (a mean of 6.7 embryos per mouse female) and 23 oocytes. The results from embryos collection are presented in table 1.

**Table 1**

Total number of oocytes and embryos recovered

Donor females (n)	Recovered embryos		Recovered oocytes	
	Total	$\bar{X}$	Total	$\bar{X}$
10	67	6.7	23	2.3

In the table 2 and 3 are presented the developmental stages of the recovered embryos and oocytes status.

**Table 2**

## Status of the recovered oocytes

Oocytes recovered (n)	Oocytes status			
	with cumulus ooforus		without cumulus ooforus	
23	n	%	n	%
	13	56	10	43

**Table 3**

## Developmental stages of the recovered embryos

Embryos recovered (n)	Developmental stages of the recovered embryos					
	2 cell		4 cell		8 cell	
67	n	%	n	%	n	%
	5	7.4	17	25.3	45	67.3

From the 67 recovered embryos 7.4% were in the 2 cells stage, 25.3% were in 4 cells stage and the majority 67.3% were in 8 cells stage, 56% form the recovered oocytes were oocytes with cumulus ooforus and 43% were oocytes without cumulus ooforus.

**Table 4**

## The recovered embryos quality

Recovered embryos (n)	Very good		Good		Satisfying		Poor	
	n	%	n	%	n	%	n	%
67	17	25.3	19	28.35	21	31.35	10	15

In table 4, is presented the quality of the recovered embryo evaluated by morphological criteria. From the total number of recovered embryos (67), 25.3% (17) were appreciated as very good quality, 28.35% (19) were classified as good quality embryos, 31.3% (21) were appreciated as satisfying quality and the rest (15%) were appreciated as poor quality. The retarded and degenerated embryos were considered as poor quality.

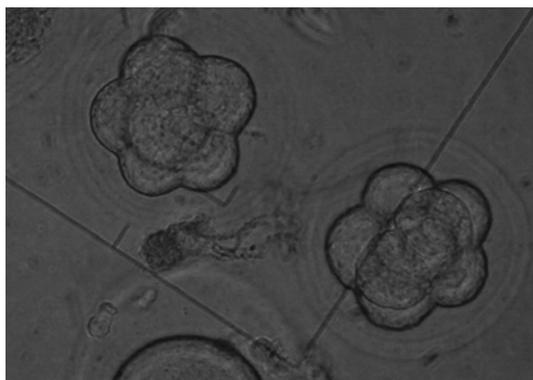
In table 5, are presented the results obtained after staining the embryos and oocytes with the two staining methods: Trypan blue and Neutral red. From the total number of recovered embryos 30 were stained with Trypan blue and from those 19 (63.3%) were evaluated as viable (uncolored) and 11 (36.7%) were evaluated as nonviable (colored in blue) and from 10 oocytes colored with Trypan blue 9 were uncolored (viable) and 1 was colored in blue (nonviable). Using Neutral red method were stained 37 embryos from which 24 (64.8%) embryos were colored in red (meaning that in those embryos was still present enzymatic activity) and were appreciated as viable and 13 (35.2%) remained uncolored and were appreciated as nonviable. Using Neutral red staining 13 oocytes were evaluated from this 9 were

colored in red and were appreciated as viable and 4 remained uncolored and were considered as nonviable.

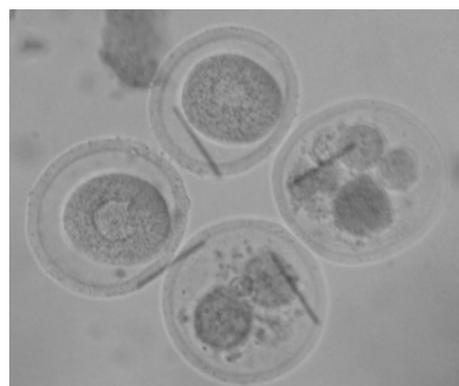
**Table 5**

Assessment of embryos and oocytes using Trypan blue exclusion and Neutral red staining

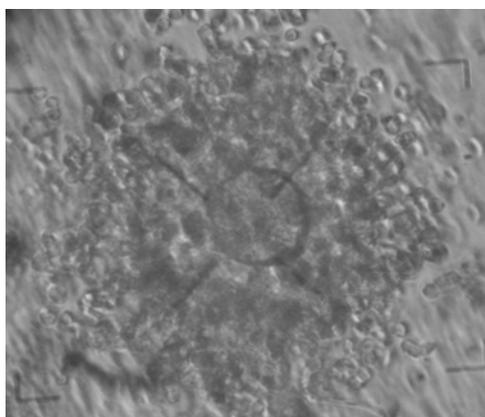
Staining methods	Specification	N	Viability			
			Viable		Nonviable	
			n	%	n	%
Trypan blue	Oocytes	10	9	90	1	10
	Embryos	30	19	63.3	11	36.7
Neutral red	Oocytes	13	9	62.3	4	37.7
	Embryos	37	24	64.8	13	35.2



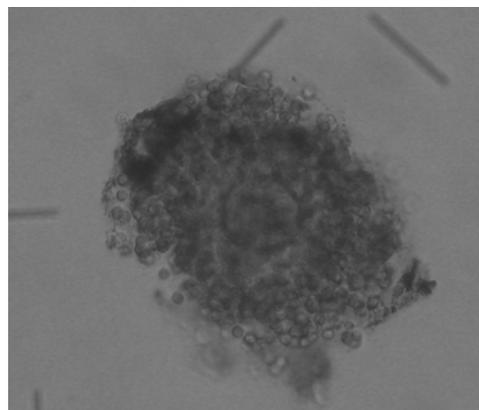
**Figure 1** Viable embryos assessed by Trypan blue exclusion



**Figure 2** Viable oocytes and nonviable embryos assessed by Trypan blue exclusion



**Figure 3** Oocyte before Neutral red staining



**Figure 4** Viable oocyte stained with Neutral red

Percentages of viable and nonviable embryos assessed by the two staining methods, seems to be very similar and both have shown a higher sensitivity in evaluation comparing with the morphological criteria method. This might indicate way the pregnancy rates after transferring the embryos assessed by morphological criteria are usually low.

### Conclusions

1. Using Trypan blue exclusion were evaluated 30 embryos from which 19 (63.3) were classified as viable and 11 (36.7) were classified as nonviable. By Neutral red staining were evaluated 37 embryos from which 24 (64.8) were considered viable and 13 (35.2) were considered nonviable. Using Trypan blue exclusion were stained 10 oocytes from which 9 remained uncolored and were considered viable and 1 was stained in blue and was considered nonviable and using Neutral red 13 oocytes were stained from which 9 were evaluated as viable and 4 as nonviable.

2. Is obviously that staining methods allow differentiating the nonviable embryos even if these embryos are still presenting good morphological characteristics, but still remains a less exploded area – to establish how staining methods influence future embryos development.

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*În prezent există mai multe metode de evaluarea a calității embrionilor însă, cea mai răspândită metodă de apreciere este cea după criterii morfologice. În acest experiment am urmărit testarea unor metode de colorare a embrionilor și ovocitelor de șoarece. Embrionii au fost recoltați de la femele de șoarece la 72 de ore de la montă. Embrionii obținuți au fost apoi apreciați din punct de al viabilității după criterii morfologice și apoi prin colorarea cu Trypan blue și Neutral red. Prin colorarea cu Trypan blue au fost evaluați 30 de embrioni din care 19 (63,3%) au fost considerați viabili și 11 (36,7%) au fost apreciați ca neviabili iar prin colorare cu Neutral red din 37 de embrioni, 24 (64,8%) de embrioni au fost considerați viabili iar 13 (35,2%) au fost apreciați ca și neviabili. Ovocitele recoltate au fost de asemena evaluate astfel 10 au fost colorate cu Trypan blue din care 9 au fost apreciate ca viabile și 1 ca neviabilă iar cu Neutral red din 13 ovocite testate 9 au fost evaluate ca viabile iar 4 ca neviabile.*

**Cuvinte cheie:** embrioni, viabilitate, colorare cu Trypan blue, colorare cu Neutral red