CHARACTERIZATION OF RABBIT FOLLICULAR OOCYTES AND THEIR ABILITY TO MATURE *IN VITRO*

CARACTERIZACIÓN DE LOS OOCITOS FOLICULARES DE LA CONEJA Y SU CAPACIDAD PARA MADURAR *IN VITRO*

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Additional keywords

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Palabras clave adicionales

IVM. Meiosis. Aspiración folicular. Sección folicular.

SUMMARY

The present study was undertaken to characterize the cellular morphology, meiotic configuration, and the ability to mature *in vitro* of rabbit follicular oocytes in relation to the method of recovery.

Oocytes (n= 433) were recovered from the ovaries of unstimulated does using either aspiration or follicle slicing and subsequent rupture. Oocytes were classified according to the characteristics of the cumulus cells and ooplasm into one of five groups: (A) sourrounded by layers of cumulus cells and intact cytoplasm; (B) naked and with intact cytoplasm; (C) with degeneration in the cumulus cells; (D) with degeneration in the cytoplasm, and (E) with degeneration in both, cytoplasm and cumulus-cells.

In experiment 1, oocytes were morphologically classified into five groups, and fixed and stained with acetic-orcein to asses their nuclear status. In experiment 2, oocytes were recovered (n= 96 and n=105, for aspiration and slicing, respectively), classified as above, and they were then matured *in vitro* in Brackett medium for 12 h. At the end of culture oocytes were assessed for maturation by staining with acetic-orcein. When aspiration was used, 105 oocytes were recovered from 146 follicles, whereas 127 oocytes were recovered by rupturing 139 follicles. The proportions of the five types of recovered oocytes were similar in the two methods of oocyte collection. Only oocytes without morphological degeneration (A and B types) showed high values of normal germinal vesicle configuration and nuclear maturation. The maturation rates using aspiration were 83.3 p.100 (type A), 52.3 p.100 (type B), 7.6 p.100 (type C), 5.8 p.100 (type D), and 0 p.100 (type E), while using follicular slicing were: 87.8 p.100 (type A), 47.3 p.100 (type B), 5.2 p.100 (type C), and 0 p.100 (types D and E).

In conclusion, (i) follicular aspiration and follicle slicing offered a similar quality in the population of the obtained oocytes. Follicle rupture can yield more number of oocytes than aspiration, but follicle aspiration has the advantage of higher speed operation; (ii), oocytes without morphological degeneration presented higher maturation rates (87.8 p.100, $p<0.01$) than degenerate oocytes, and

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(iii) the extremes in investment and ooplasm appareance were the only obvious indicators of an oocyte's potential to mature *in vitro*.

RESUMEN

El presente estudio ha sido realizado para caracterizar la morfología celular y meiótica de oocitos foliculares de coneja, así como su capacidad de madurar *in vitro* de acuerdo al método de obtención de los mismos.

Los oocitos (n=433) se recogieron de ovarios de conejas mediante (a) aspiración del contenido de los folículos o, (b) sección y ruptura de los mismos. Los oocitos así obtenidos se clasificaron en cinco grupos: (A), rodeados de células del cúmulo; (B) desnudos, pero con citoplasma intacto; (C) con degeneración en las células del cúmulo; (D) con degeneración en el citoplasma y (E) con degeneración en ambas localizaciones.

En el experimento 1, los oocitos se clasificaron en estos cinco grupos y fueron fijados y teñidos con orceína acética para mostrar su estado nuclear. En el experimento 2, los oocitos obtenidos (n=96 y n=105, con aspiración y sección, respectivamente), fueron clasificados de igual manera a lo expuesto anteriormente y se cultivaron *in vitro* durante 12 h en medio de cultivo de Brackett. Al final de este periodo, los oocitos se fijaron y tiñeron también con orceína acética. Mediante la aspiración del contenido folicular se recogieron 105 oocitos procedentes de 146 folículos, mientras que con disección, se recogieron 127 oocitos de 139 folículos. Las proporciones de los cinco tipos de oocitos recogidos fueron similares en ambos métodos. Sólo los oocitos sin alteraciones morfológicas (tipos A y B) mostraron una configuración nuclear normal de vesícula germinal y maduraron correctamente en un alto porcentaje. Los porcentajes de maduración nuclear al utilizar la aspiración fueron de 83,3 p.100 (tipo A), 52,3 p.100 (tipo B), 7,6 p.100 (tipo C), 5,8 p.100 (tipo D) y 0 p.100 (tipo E); mientras que mediante sección y ruptura folicular fueron: 87,8 p.100 (tipo A), 47,3 p.100 (tipo B), 5,2 p.100 (tipo C) y 0 p.100 (tipos D y E).

En conclusión, la sección folicular provee de más oocitos, pero la aspiración se realiza más rápidamente. Los oocitos sin degeneración aparente maduran en un mayor porcentaje que los que sí la presentan. Finalmente, las características del citoplasma y del cúmulo que rodea los oocitos, parecen ser los únicos indicadores de la capacidad potencial de maduración *in vitro* de los oocitos de coneja.

INTRODUCTION

Research on mammalian oocytes and embryos has become increasingly important in basic and applied reproductive physiology. For various reasons rabbit oocytes are widely used as *in vitro* model in these experimental procedures; the rabbit is one of the few species in which ovulation is induced by mating, and allows adjustments of animal work to experimental conditions as well as to usual working hours (Fischer and Meuser-Oderkirchen, 1988).

On the other hand, mammalian oocytes in non-atretic follicles are arrested at the germinal vesicle stage (GV) of the first meiotic division, until the preovulatory surge of gonadotropins initiates resumption of meiosis (reviewed by Wassarman, 1988). Follicle-enclosed oocytes cultured *in vitro* remain immature until they are exposed to luteinizing hormone or human chorionic gonadotropin (Liberman *et al.*, 1976; Kobayashi *et al.*, 1981). The ability to mature oocytes *in vitro* from ovarian follicles would greatly increase the yield of oocytes available for fertilization. Alterations in oocyte maturation represent a serious loss of efficiency in the IVM-IVF treatment cycle since it is associated with a low

fertilization rate (Marrs *et al.*, 1984; Zenzes *et al.*, 1990), polyspermic fertilization (Hunter *et al.*, 1976), poorer embryo quality (Haines and Emes, 1991) and lower pregnancy rate (Veeck, 1985). Additionally, the extent of the investment around the oocytes, integrity of cytoplasm, and stage of nuclear development might be used to identify the oocytes most likely to mature *in vitro* in some species (Tsafriri and Channing, 1975; Leibfried and First, 1979). Although morphology and characterization of immature oocytes obtained from large animals has been described in several reports, to date however, the obtained oocytes from rabbit ovaries has not previously been documented sufficiently. Aspiration of follicular fluid with oocytes using a syringe and needle is a commonly used method of recovering immature oocytes (Yoshimura *et al.*, 1989; Hosoi *et al.*, 1989). This rather simple collection method recovers as many as 70 p.100 of the follicular oocytes (Lebfried and First, 1979; Katska, 1984). Therefore, it is possible that good oocytes for *in vitro* culture are not recovered at all.

To substantiate this theory, two methods of oocytes recovery were compared in the present study: aspiration, and slicing and subsequent rupture of follicles. Comparison was done on the basis of recovery rate, cellular and nuclear morphology of the collected oocytes, and their ability to mature *in vitro*.

MATERIAL AND METHODS

ANIMALS

Sexually mature New Zealand White x Californian rabbits (3.5-4.5 kg body weight) were used. The animals were housed individually in metal cages on perforated sheets of dimensions 32 cm x 52 cm in air-conditioned rooms (25°C, 45 p.100 relative humidity) under a 16 h light-8 h dark cycle (average light intensity, 45±10 lx; variation range from 10 to 100 lx depending on the flat deck). Artificial light was on from 8.00 h to 24.00 h. Pelleted commercial diet (Lab Rabbit Chow, Purina Mills Inc: 16.2 p.100 protein, 2.5 p.100 fat, and 13.5 p.100 crude fiber) was restricted to about 125 g once daily. Water was supplied *ad libitum*. Animals were kept for at least 16 days before treatment. Does were killed with intravenous pentobarbitone sodium, and their ovaries were removed immediately.

OOCYTE RECOVERY, CLASSIFICATION AND MATURATION *IN VITRO*

The ovaries were placed in 5 ml Brackett's defined media sterilized by filtration (Brackett and Oliphant, 1975) in a 60 mm petri dish and the number of large preovulatory follicles (>1 mm in diameter) was recorded. Ovaries were excluded from further study if they appeared to be immature or if 50 p.100 or more of surface follicles appeared hemorrhagic. Selected follicles were either aspirated or sliced under a dissecting microscope. These follicles were chosen in the present study due to the fact that follicles smaller than 1 mm in diameter contain oocytes which are not competent for achieving meiosis (Jelinkova *et al.*, 1994). Aspiration was performed in one of the ovaries with a 25-G needle. Slicing was performed in the other ovary with an iris knife to release the follicular contents. Collected

oocytes from individual ovaries were kept separate in 35 mm, sterile, disposable Petri dishes using an orally controlled micro-pipette. Petri dishes contains 1.0 ml of Brackett's medium, wich had been equilibrated at 37°C in an atmosphere of 5 p.100 CO_2 in air and 100 p.100 humidity for 2 h, and given five washes before classification.

After recovery, oocytes were examined under dissecting microscope and classified according to the morphology of surrounding cumulus cells and cytoplasm into one of five groups: (A), oocytes with intact cytoplasm, surrounded by compact cumulus layers; (B), oocytes with intact cytoplasm, naked; (B), oocytes with degenerative changes in the cumulus cells; (D), oocytes with degenerative changes in the cytoplasm, and (E), oocytes with degenerative changes in both, cumulus and cytoplasm. Oocytes were scored as degenerate if they showed vacuolization, citolysis, or loss of spherical shape.

Finally, oocytes were cultured under 1.0 ml of Brackett's medium in 35 mm Petri dishes at 37°C under 5 p.100 CO_2 in air and 100 p.100 humidity for 12 h. The pH of the medium was adjusted to 7.3-7.4 prior to use in culture.

NUCLEAR EVALUATION

At the time of recovery in experiment 1, and at the end of the culture period in the experiment 2, the oocytes were fixed and stained to ascertain their meiotic stage (Lorenzo *et al.,* 1994). Brielfy, cumulus cells were removed with hyaluronidase (200 UI/ml) and mechanically stripped using a fine-bore pipette. The oocytes were then pipetted onto a slide. A coverslip spotted with a paraffin-vaseline (10:1) mixture at each corner was placed directly over the center of the drop containing oocytes. Fixation of oocytes was carried out by placing the slides in acetic acid-ethanol (1:3) for 24 h and staining with aceto-orcein (2 p.100 orcein in 60 p.100 acetic acid) for two minutes. Nuclear maturation was evaluated under phase-contrast microscope at 200x and 500x magnifications. Oocytes were assessed for stage of maturity as previously described (Kobayashi *et al.,* 1981). Oocyte maturation was expressed as the percentage of oocytes which had achieved metaphase II stage. Oocytes were also assessed for intact germinal vesicle (GV, in which nuclear membrane and nucleolus were evident), germinal vesicle breakdown (GVBD), and degenerative changes (if they showed dispersed chromosomes, necrosis or vacuolization).

Experiment 1. The initial experiment was designed to determine the differences in the method of recovery oocytes. Comparison was done on the basis of recovery rate, cellular morphology, and nuclear status of the different types of obtained oocytes at recovery time. These oocytes were morphology characterized, and fixed and stained to ascertain their meiotic stage, as described above.

Experiment 2. This experiment tested the ability of the five morphological groups of oocytes removed either with follicle aspiration or slicing, to mature *in vitro*. As is it described, the oocytes were fixed and stained to ascertain their nuclear stage at the end of the culture period.

STATISTICAL ANALYSIS

Experiment 1 was repeated eight times with different batches of ovaries. Experiment 2 was replicated five times. Two female rabbits acted as ovary donors on the same day for experiment 1, while oocytes from three does were used on the same day for experiment 2. Because the number of oocytes cultured in each replicate experiment was small, and the frequency of oocytes in each morphologic category even smaller, meaningful Chi-square test for homogeneity could not be done. However, replicate results appeared homogeneous and were therefore pooled. The significance of treatment effects was determined by Catmod procedure of Statistical Analysis System (SAS/STAT, 1987). Percentages were compared using the Chi-square test (Yates, 1949). Only P values less than .05 were considered significant.

RESULTS

A total of 433 oocytes were obtained and stained. **Table I**, shows the proportions of the obtained follicular oocytes according to the method of recovery. **Tables II** and **III** depict the

Table I. *Differences between methods of recovery follicular oocytes in female rabbits.* (Diferencias entre los métodos para recoger oocitos foliculares en la coneja).

Method	Ovaries used	Follicles selected	Oocytes obtained	Follicles/ ovary*	Oocytes/ ovary*	Recovery rate[1] (p.100)
Aspiration	16	146	105	9.1 ± 0.5[a]	6.6 ± 0.6[a]	71.9[a]
range				6-14	4-14	47.1-100
Slicing	16	139	127	8.4 ± 0.6[a]	7.9 ± 0.8[a]	91.3[a]
range				4-14	3-15[2]	58.3-100

	Percentage of oocytes in different morphological classes*				
	A	B	C	D	E
Aspiration	71.4 ± 4.6[a]	9.5 ± 0.9[a]	6.6 ± 1.0[a]	4.8 ± 0.2[a]	7.6 ± 0.6[a]
observed/total	75/105	10/105	7/105	5/105	8/105
Slicing	74.1 ± 5.8[a]	6.4 ± 0.8[a]	6.4 ± 0.8[a]	6.6 ± 0.7[a]	7.9 ± 0.8[a]
observed/total	103/139	9/139	9/139	7/139	11/139

* Values are in percentage ± SEM

[1] Expressed as recovered oocytes / selected follicles x 100

[2] Two oocytes were found in one follicle

[a] There was no statistical difference between the two methods (p>0.05) by Chi-square test.

distribution of the obtained meiotic stages in the oocytes at time of recovery, and at the end of the maturation period for each recovery method. In the experiment 1, 146 follicles were aspired and 139 were sliced from a total of 32 ovaries. **Table I** shows the total number of oocytes obtained and the average per ovary in both methods, aspiration and slicing of ovarian follicles. No statistically significant differences in both, the number of recovered oocytes per ovary and the recovery rate (oocytes obtained/follicles selected x100), were found between the two methods. Although the proportion of the obtained naked oocytes (type B) was higher in aspiration than in slicing (9.5 ± 0.9 p.100 *vs* 6.4±0.8 p.100), the difference was not significant (p>0.05). The number of oocytes in the other morphological classes are exposed in **table I**. The comparision between results obtained for each type of oocytes, by follicular aspiration or slicing, did not present differences statistically significant.

Tables II and **III** show that only oocytes without degenerative changes in cytoplasm or cumulus (types A and B) presented the highest percentages of normal meiotic configuration (GV stage) at recovery time. The oocytes from types C and D presented high percentages of nuclear degeneration, while those of

Table II. *Nuclear stages of rabbit oocytes obtained at the time of recovery.* (Estadios nucleares de oocitos de coneja obtenidos en al momento de su obtención).

Type of oocyte	Follicular aspiration* (observed/total)				Follicular slicing and rupture* (observed/total)			
	n	GV	GVBD	Deg	n	GV	GVBD	Deg
A	75	85.3 ± 6.1^{a} (65/75)	8.0 ± 0.5^{a} (6/75)	5.3 ± 0.6^{a} (4/75)	94	92.9 ± 7.2^{a} (78/94)	10.6 ± 1.3^{a} (10/94)	6.3 ± 0.5^{a} (6/94)
B	10	70.0 ± 6.8^{a} (7/10)	10.0 ± 0.0^{a} (1/10)	20.0 ± 0.1^{a} (2/10)	7	71.4 ± 5.9^{a} (5/7)	14.2 ± 0.0^{a} (1/7)	14.2 ± 0.0^{a} (1/7)
C	7	14.2 ± 0.0^{b} (1/7)	14.2 ± 0.0^{a} (1/7)	42.8 ± 0.3^{b} (3/7)	8	12.5 ± 1.7^{b} (2/8)	12.5 ± 0.0^{a} (1/8)	62.5 ± 6.2^{b} (5/8)
D	5	20.0 ± 0.0^{b} (1/5)	20.0 ± 0.0 ^{a} (1/5)	60.0 ± 0.7^{b} (3/5)	7	14.2 ± 0.0^{b} (1/7)	14.2 ± 0.0^{a} (1/7)	71.4 ± 9.0^{b} (5/7)
E	8	0.0^{b} (0/8)	0.0^{a} (0/8)	100.0^{b} (8/8)	11	0.0^{b} (0/11)	0.0^{a} (0/11)	100^{c} (11/11)

* Values are in percentage ± SEM. GV: Germinal Vesicle stage. GVBD: Germinal Vesicle breakdown. Different superscripts within values in the same column indicate statistical differences: a vs b, p<0.05; b vs c, p<0.05; a vs c, p<0.01, by Chi-square test.

Table III. *Nuclear stages of rabbit oocytes at the end of the* in vitro *maturation period.* (Estadios nucleares de oocitos de coneja al término de su periodo de maduración *in vitro*).

Type of oocyte	Follicular aspiration* (observed/total)				Follicular slicing and rupture* (observed/total)			
	n	GV	Matured[1]	Deg	n	GV	Matured[1]	Deg
A	30	3.0 ± 0.0[a] (1/30)	83.3 ± 7.2[a] (25/30)	13.0 ± 0.2[a] (4/30)	41	4.8 ± 0.1[a] (2/41)	87.8 ± 7.0[a] (36/41)	7.3 ± 0.5[a] (3/41)
B	21	28.5 ± 0.1[b] (6/21)	52.3 ± 6.1[b] (11/21)	19.0 ± 3.3[a] (4/21)	19	36.8 ± 4.2[b] (7/19)	47.3 ± 3.4[b] (9/19)	15.7 ± 2.2[a] (3/19)
C	13	23.0 ± 2.0[b] (3/13)	7.6 ± 0.0[c] (1/13)	61.5 ± 4.8[b] (8/13)	19	31.5 ± 4.2[b] (6/19)	5.2 ± 0.6[c] (1/19)	63.1 ± 4.2[b] (12/19)
D	17	35.2 ± 3.0[b] (6/17)	5.8 ± 0.0[c] (1/17)	58.8 ± 5.1[b] (10/17)	14	35.7 ± 0.1[b] (5/14)	0.0[c] (0/14)	62.2 ± 3.7[b] (9/14)
E	15	6.6 ± 0.0[a] (1/15)	0.0[c] (0/15)	93.3 ± 8.1[c] (14/15)	12	0.0[a] (0/12)	0.0[c] (0/12)	100.0[c] (12/12)

* Values are in percentage ± SEM. GV: Germinal Vesicle stage.
[1]Matured are oocytes in metaphase II stage at the end of culture.
Different superscripts within values in the same column indicate statistical differences: a vs b, $p<0.05$; b vs c, $p<0.05$; a vs c, $p<0.01$, by Chi-square test.

type E (with degeneration in both, cumulus and cytoplasm) showed 100 p.100 of nuclear degeneration. There were no statistical differences ($p>0.05$) in the obtained meiotic configurations between the methods of oocyte recovery for each type of recovered oocytes.

In the experiment 2, these were no differences in maturation rate between oocytes recovered either by aspiration or slicing. In both methods, the oocytes with cumulus layers and no degeneration changes (type A) always matured in higher proportion than naked oocytes ($p<0.05$), and specially than degenerate-type oocytes C, D, and E ($p<0.01$). Finally, highly significant relation was found between oocytes with high percentages of intact nuclear chromatin (GV stage) at recovery time, and their ability to mature *in vitro*, for oocytes recovered either by aspiration or slicing. For example, by aspiration method, only 7.6 p.100 and 5.8 p.100 of oocytes named partially degenerated at time recovery of oocytes (C and D types), and 0.0 p.100 of oocytes termed fully degenerated (type E) matured *in vitro*, compared to 83.3 p.100 of those without degeneration and with cumulus layers (type A). Similar results were found by slicing method, as it is shown in **table II** and **III**.

DISCUSSION

Characterization of morphological appearance and determination of meiotic stage of immature oocytes are important parameters to improve *in vitro* gamete procedures such as IVM-IVF. The characteristics of the oocytes originating from antral follicles have been described in different species, as the mice (Erickson and Sorensen, 1974) and cow (Rajakoski, 1960; Leibfried and First, 1979). However, though some studies have been published about capacity of rabbit oocytes to mature *in vitro* (Chang, 1955; Bae and Foote, 1975; Hosoi *et al.*, 1989; Jelinkova *et al.*, 1994), neither the morphology of the antral follicle oocytes nor the peculiarities of the two methods commonly used to obtain them, have been characterized, to date.

The revised studies indicate that through slicing is collected a greather number of oocytes than with follicular aspiration (Arlotto *et al.*, 1990). The reported low recovery rate for aspiration method, may be due to the dificulties in separating the oocyte from follicular granulosa cells. According to related in previous papers, aspiration of the follicles could tempt a partial loss of cumulus cells also, as occurs in cattle follicle aspiration also (Katska, 1984). However, in the present study in the rabbit, there were no significant differences in the proportions of oocyte-type recovered in both methods, indicating that cumulus cell-layers surrounding oocytes are not damaged during aspiration. This is due, probably, to the use of a needle diameter sufficiently broad as not to damage the cumulus-layers of the oocyte. Therefore, the obtained results indicate that both methods can yield a great number of acceptable quality oocytes for *in vitro* studies in the rabbit; it should be stressed that aspiration of follicles *in situ* is the more practical of the two described methods. It enables a relatively quick collection of a significant quantity of oocytes, and has the advantage of an speed operation (reducing the possible alterations of pH, temperature, and bacterial contamination of culture media). On the other hand, rupturing isolated follicles gives an almost 100 p.100 recovery rate. Therefore, the best method of oocyte recovery depends on the number of ovaries to process, and the experience of the team that accomplishes it.

On the other hand, all the revised authors agree that many factors affect oocyte maturation and fertilization. The source and quality of the oocytes are the first factors affecting it all of these *in vitro* procedures. In studies in other animal species (e.g. cattle), the oocyte maturation rate of these oocytes obtained by slicing was lower than aspiration (Arlotto *et al.*, 1990; Lonergan *et al.*, 1991). However, in the present study, the proportions of matured oocytes were similar to that reflected by authors that used either aspiration (Hosoi *et al.*, 1989) or slicing method (Smith *et al.*, 1978); moreover, maturation rate of type A-oocytes after 12 hours in culture was similar to that in previous reports (Hosoi *et al.*, 1989; Jelinkova *et al.*, 1994). Therefore, differences with the above reported studies could be due to the use in the above reported studies of smaller follicles for slicing. These follicles contain oocytes which have not completed their growth, and are uncompetent to achieve maturation

(Arlotto *et al.,* 1990). Moreover, the morphological characteristics in the C, D, and E oocyte-type were indicative of oocyte degeneration; if the ability of oocyte to mature *in vitro* is a function of oocyte degeneration, selection of oocyte samples based on morphological appearance should allow an *a priori* assessment of sucessful development *in vitro*. Relation between oocyte appareance and nuclear intact chromatin was demonstrated in the experiment 1; also, relation between oocytes with or without degenerative changes and their capability to mature *in vitro* was observed in experiment 2. Therefore, the obtained results of these experiments suggest that the presence of cellular investment, intact cytoplasm, and intact nuclear chromatin (GV stage) may be the only determinants of the rabbit oocyte's ability to mature *in vitro* correctly.

In the present study, the oocytes without degeneration changes were either surrounded or naked of cumulus cells at recovery time of oocytes. However, the obtained results in maturation were higher in oocytes surrounded of cumulus cells than naked. This inability of nude oocytes to mature *in vitro* has previously been show for both, porcine and bovine oocytes (Fukui *et al.,* 1980; Xu *et al.,* 1986). This demonstrate that maturation action requires the presence of cumulus cells, by which a positive stimulus for nuclear maturation is transferred to the oocyte (Xu *et al.,* 1986; Downs *et al.,* 1988).

The whole results of these experiments showed that it is important to classify oocytes according to integrity of cumulus cells and cytoplasm criteria before to begin *in vitro* procedures in the rabbit. In order to utilize oocytes effectively, oocytes without morphological and nuclear degeneration should be selected to undergo *in vitro* procedures such IVM-IVF. We trust that henceforth these values may serve as basic or standard points of relevance for any future studies concerning to the physiology of reproduction of the rabbit.

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