IN VITRO FERTILIZATION AND ITS RELATED TECHNIQUES

FECUNDAȚIA *IN VITRO* ȘI TEHNICI CONEXE ACESTEIA

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ABSTRACT

Since starting activity in 2014 in *Assisted Reproduction,Cellular and Molecular Biology Laboratory* from "*Horia Cernescu Research Laboratories Complex*" Timișoara, the main topics represented research (study of COC's quality during *in vitro* fertilization technique). Because it is known that the competence of the oocyte to yield a blastocyst within *in vitro* fertilization production is probably the most important step that can influence the success of this technique, we studied this aspect in different species. *In vitro* maturation (IVM) of bovine and swine COC's was done based on their morphological aspects (I, II and III class) in TCM 199 HEPES modification media with 10% ECS and 15 µl FSH. IVM of queen 1-st class COC was done after Wlodarczyk's protocol. Bovine IVF technique was using Parrish's protocol. Oocytes nuclear maturation was evaluated using aceto-orcein 1% and Hoechst 33342 staining. The genes expression was evaluated by qPCR.

Bovine COCs quality had a positive effect on cumulus expansion rate based on morphological aspects observed after maturation, irrelevant when FSH was added. Orcein staining revealed that from 75 bovine class I and II COC's maturated *in vitro*, 7.14% and 9.52 % were in MI, respectively MII in group with FSH and 6.06% and 9.09% in MI and MII in group without FSH, the rest of the oocytes being in GV and GVBD stages.Similar results we observed also on sow oocytes maturated in medium with cysteine, a precursor of the antioxidant glutation (GSH): in class CI 63,15% from COCs showed cumulus expansion, in class CII, 52.94% and 14.81% in class CIII and without cysteine: in class CI 58.82%, in class CII 45% and in class CIII 20.83% and the expression of Bcl2 was almost double in cumulus cells maturated in media with cysteine, suggesting that antioxidant supplementation can increase the viability of the oocyte. Evaluating the nuclear status of queen oocytes by Hoechst 33342 staining procedure at 30 h after IVM, we observed only 14.28% oocytes with cumulus expanded, from which 5.71 % in GVBD; 2.85 % in M II and the rest of the oocytes contained unidentifiable chromatin. After bovine IVF was done on 520 COC, from which 163 COC presented sign of maturation; after IVM we obtained 37 embryos (22.69%) with different number of blastomers: 16 had 2 cells, 12 with 4 cells, 5 with 8 cells and 4 had reached blastocyst stage in the seventh day after fertilization.

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REZUMAT

De la începutul activității Laboratorului de *"Reproducere asistată, Biologie celulară și moleculară și Proteomică"* din cadrul *"Complexului de Laboratoare de Cercetare Horia Cernescu"* din Timișoara, în anul 2014, principalele teme abordate au vizat domeniul cercetării (calitatea ovocitelor folosite la fecundația *in vitro*). Deoarece competența unei ovocite de a ajunge blastocist în timpul fecundației *in vitro* este probabil cel mai important lucru care poate influența reușita acestei tehnici, am studiat acest aspect la diferite specii.

Maturarea *in vitro* a complexelor ovocită-cumulus (COC) de bovine și suine a fost realizată în funcție de aspectele morfologice (clasa I-a, II-a, III-a) în mediu TCM199 Hepes modificat cu 10%ECS și 15 µl FSH. Maturarea in vitro a COC clasa I-a de pisică a fost realizată după protocolul lui Wladarczyk. Tehnica fecundației *in vitro* (FIV) la bovine a fost realizată după protocolul lui Parrish. Evaluarea maturării nucleare a ovocitelor a fost făcută prin tehnici de colorare: orceină-acetică 1% și Hoechst 33342. Expresia genică a fost evaluată prin tehnica qPCR. Calitatea ovocitelor de bovine a exercitat un efect pozitiv asupra gradului de expansiune - evaluat pe baza aspectelor morfologice observate după maturare, fără o influență semnificativă a FSH-ului, iar după utilizarea tehnicii de colorare cu orceină pe 75 COC de bovine clasa I-a și a II-a maturate *in vitro* s-a observat 7.14%, respectiv 9.52% erau în stadiul MI, respectiv MII în grupul unde s-a adăugat FSH și 6.06% în MI, respectiv 9.09% în MII în grupul cu FSH, restul ovocitelor fiind în stadiile de veziculă germinală (GV) și de rupere a veziculei germinale (VGBD). Rezultate similare s-au observat și la ovocitele de scroafă maturate în mediu suplimentat cu cisteină, un precursor al glutationului (GSH): 63.15% ovocite cls. I au prezentat expansiunea cumulusului, 52.94% - cls. a II-a și 14.81% - cls. a III-a, iar în mediul fără cisteină: 58.82% dintre cele de cls. I-a, 45% din a II-a și 20.83% din a III-a. Expresia genei BCL2 a fost aproape dublă în celulele cumulus ale ovocitelor maturate în mediul suplimentat cu cisteină, ceea ce indică o creștere a viabilității ovocitelor. Examinarea statusului nuclear al ovocitelor de pisică, colorate cu Hoechst 33342, la 30h după maturarea *in vitro* a relevat: 14.28% dintre ovocite aveau cumulus expandat, dintre care 5.71% erau în stadiul de GVBD; 2.85%-MII, iar restul aveau cromatina nedetectabilă. După realizarea fecundației in vitro pe 520 COC de vacă, dintre care 163 COC prezentau semne de maturare după prima etapă IVM, s-au obținut 37 embrioni (22.69%): 16 erau cu 2 celule, 12 cu 4 celule, 5 cu 8 celule și 4 embrioni au atins stadiul de blastocist.

Assisted reproduction techniques (ART) are used both for practical and research purposes in the majority of species. Of all species, in bovine the development is greatest. Bovine artificial insemination (AI) is commonly used on large-scale, in 1998 worldwide were 648 semen collection centres registered and 1635 semen banks with more than 40000 bulls and 264 million doses of semen produced (50% in Europe, 27% in the Far East and 16% in North America) with more than 20 milions doses exported [20]. Among the beneficial effects of AI, we can include: enhancing the effectiveness of the use of genetic values bulls, safer animals and farmers, lowering costs for maintenance of males, decreased risk of transmission of diseases etc. [23].

With a remarkable progress, another technique of assisted reproduction is used:*in vitro* fertilization (IVF). According to statistics of the International Embrio Transfer Society (IETS), in the year 2012, 443,533 of bovine embryos were obtained through IVF, with a noticeable increase in relation to 2007, when it were reported a number of 245,000 of bovine embryos [22].

Swine assisted reproduction presents a major interest for biomedical research, in obtaining transgenic pigs as potential donors in xeno-transplantation. Since this is achieved through techniques as nuclear transfer/ cloning or pronucleus micro-injection, where are used matured oocytes and embryos in early stages, the stage where they are obtained is very important [18]. Also swine oocytes have become an important model for the study of molecular control of the meiotic cell cycle and the transduction signal during fertilization, including in human species, since it suggests that rodents are atypical in terms of regulatory mechanisms of oocyte maturation and fertilization [18].

With regard to feline reproduction, although it focuses more on controlling breeding of domestic cats populations without owner, however ART are used in this species for preservation of all the 37 wild felid species classified as threatened with extinction, except the domestic cat, but also for human biomedical research [14]. Also in canine reproduction, ART has a biomedical purpose – more than half of the approximately 400 canine hereditary diseases have an equivalent human disease, among them muscular dystrophy, cardiomyopathies and prostate cancer [19]. The dog was among the first species used in research of transplantation [9].

We shall present some of the research conducted in the laboratory *Assisted Reproduction, Cellular and Molecular Biology and Proteomics Laboratory* (A.2): in experiment no. 1, we studied, by staining techniques, the influence of follicle-stimulating hormone (FSH) on maturation of bovine oocytes, in experiment no. 2, we studied the influence of cystein on *in vitro* maturation of pig oocytes by morphological examination and gene expression, in experiment no.3, we studied in vitro maturation of cat oocytes, while in experiment no.4 we realized several time bovine IVF.

MATERIALS AND METHODS

Bovine (n=94) and sows ovaries (n=58) were collected from local slaughterhouses and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep), at 33-35^{0}C within two hours. Cat ovaries (n=6) were recovered after ovariohysterectomy performed at Clinic of Reproduction, Obstetrics and Veterinary Gynecology from Faculty of Veterinary Medicine Timișoara.

Handling medium for COC was DPBS (D-8662) supplemented with 100 µl Pen/Strep (17-602F, Lonza); 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021). Bovine and swine COCs were collected by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe and from cat ovaries by slicing procedure.

Morphological classification of COCs from all 3 species was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^{0}C) as follows : *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous; dark and uniform cytoplasm in cats COC), *IInd class*– CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class*– CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and with irregular shrunken cytoplasm). Only COC grade I was selected for IVM in cats.

For the first experiment, the bovine oocytes (171 COC from 20 ovaries) were maturated in a medium prepared in our laboratory after Parrish et al. (1986) protocol with minor modifications: TCM 199 HEPES modification media, (M2520) with 10% ECS and with or without sheep FSH (F8174). Pools of 8-10 COCs were maturated in 50µl TCM-199 with/without 0.5 µl FSH (0.88 µg/ml) in 35 mm Petri dishes (Greiner Bio-One, Germany) covered with mineral oil at 38.5^{0}C in 5% CO_2humidified air atmosphere for 24h. After 24h of culture, all oocytes were examined for maturation, signs as expansion and mucification of cumulus cells were observed. The oocytes were denuded using a fine capillary glass and examined for first polar bodies at

stereomicroscope (5X). All oocytes denudated were stained with 1% aceto-orcein after method of Khatun et al. (2011)[8] with minor modification (for fixing the gass cover slip, 18X18 mm, on the drops we made only two lines with Baysilone-Paste). The slides were examined under microscope (Leica DM750) for nuclear changes (40X). Oocytes were considered nuclear maturated if extrusion of the first polar body and appearance of the metaphase plate (MII) were present. For Hoechst staining, oocytes were denuded using a fine capillary glass, after that they were incubated 15 minutes in 400 µl PBS with 5 µl Hoechst and examinated with inverted fluorescence microscope (Leica DMI4000 B)(60X) in the *Laboratory of cytogenetics and molecular genetics* from *Complex of Research Laboratories "Horia Cernescu".*

For the second experiment the sow oocytes (119 COC from 58 ovaries) were maturated in a medium prepared in our laboratory: TCM 199 HEPES modification media with 10%ECS, 10 mg/ml BSA, 0.5 and/ without 0.1 mg/ml cysteine in drops of 80µl medium covered with mineral oil at 38.5^{0}C in 5% CO_2 humidified air atmosphere for 44h. After evaluation of maturation based on morphological aspects, the oocytes were denuded mecanically and the cumulus cells were prepared for total RNA isolation. Total ARN was isolated and purified from the sediment cells using *SV Total RNA Isolation System* (Promega, US) commercial kit following the producers indications. Quantity and quality of extracted RNA was assesed by measurements with *NanoDrop 8000* spectrophotometer (Thermo Scientific). From isolated RNA, the cDNA was synthesized using *First Strand cDNA Synthesis Kit* (Fermentas), following the producer indications, and oligo dT(8) primer, also provided with the kit. The cDNA obtained was used as template in qPCR reactions using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), according to provided protocol with a *Stratagene Mx3000P* (Agilent) real time PCR equipment. The primers sequences for BCL2 gene were (primer sense 5'-3'**:** GAAACCCCTAGTGCCATCAA and primer antisense 3'-5'**:** GGGACGTCAGGTCACTGAAT). For the relative quantification the Δ (ΔCt) method was used [11]. For all of the samples the number of cycles (Ct) was determined. According to this method the R (the relative ratio between the control and stressed variant) is calculated with the following formula: $R = 2^{-\Delta\Delta Ct.}$

For the third experiment, the cat oocytes (35 COC from 6 ovaries) were maturated in medium prepared in our laboratory after Wlodarczyk's protocol. Only COC grades I were cultured in maturation medium (TCM-199 Earl's medium supplemented with 25mM HEPES, 4 mg/ml bovine serum albumin, 2.20 mg/ml NaHCO3, 50 µg/ml gentamicin sulfate, 0.2 mM sodium pyruvate, 10 µg/ml FSH, 1.06 UI/ml HCG) supplemented with 0.13 mmol/L cysteine and 0.5 mmol/L ascorbic acid. Pools of 5-6 COC were maturated in 50 µl medium drops, at 39.5 ^{0}C and 5% CO_2 atmosphere, for 30h.

For the fourth experiment, the bovine oocytes (520 COC from 74 ovaries) used in 8 IVF sessions were maturated, fertilized and cultured in medium prepared in our laboratory as follow: for COC maturation - modified TCM 199 with FSH and ECS; for fertilization - modified Tyrode Lactate medium [16], for sperm preparation we used Sperm-TALP medium [2] and for embryos culture -modified TCM199 with ECS 10% exchanged at 48h interval with the same volume taken out from the drops.

RESULTS AND DISCUSSION

The morphological aspects of COC before and after *in vitro* maturation used in experiment 1, 2 and 3 are presented in Figure 1. Regarding the first experiment, we can observed that COCs quality had a positive effect on cumulus expansion rate based on morphological aspects observed after maturation, without significant effect of FSH. In class CI, 85.34% from COCs showed cumulus expansion ($p<0.76$), in class CII, 75% ($p< 0.61$) and in class III, 43.3% ($p<0.03$), similar results were observed also in oocytes cultured without FSH: in class CI, 84.03 % ($p<0.27$), in class CII, 68.06% ($p< 0.29$) and in class CIII, 33.28% ($p<0.09$). After orcein staining of 75 bovine class I and II COC's maturated *in vitro,* 7.14% and 9.52% were in MI, respectively MII in group with FSH and 6.06% and 9.09% in MI and MII in group without FSH, more than 50% were in germinal vesicle stage and germinal vesicle break down stage [13]. Hoechst 33342 is another nuclear dye that can be use in ART technology but without affecting the viability of the cells, if exposure to UV irradiation is short. In another study done by our group [5], we presented the rate of bovine oocytes that reach stage MII based on gamete maturation marker: 33.33% of class I oocytes and 24% of class II oocytes maturated in medium without FSH. Even there were no significant differences between maturation percentages in our experiment, it is known that gonadotropins have a huge impact on efficacy of most assisted reproductive technologies.

Recent studies (Assidi et al., 2013) reported, based on micro-array analysis, *in vitro* FSH stimulation on cumulus cells appears to achieve at least part of the gene expression activity after *in vivo* LH stimulation [1].

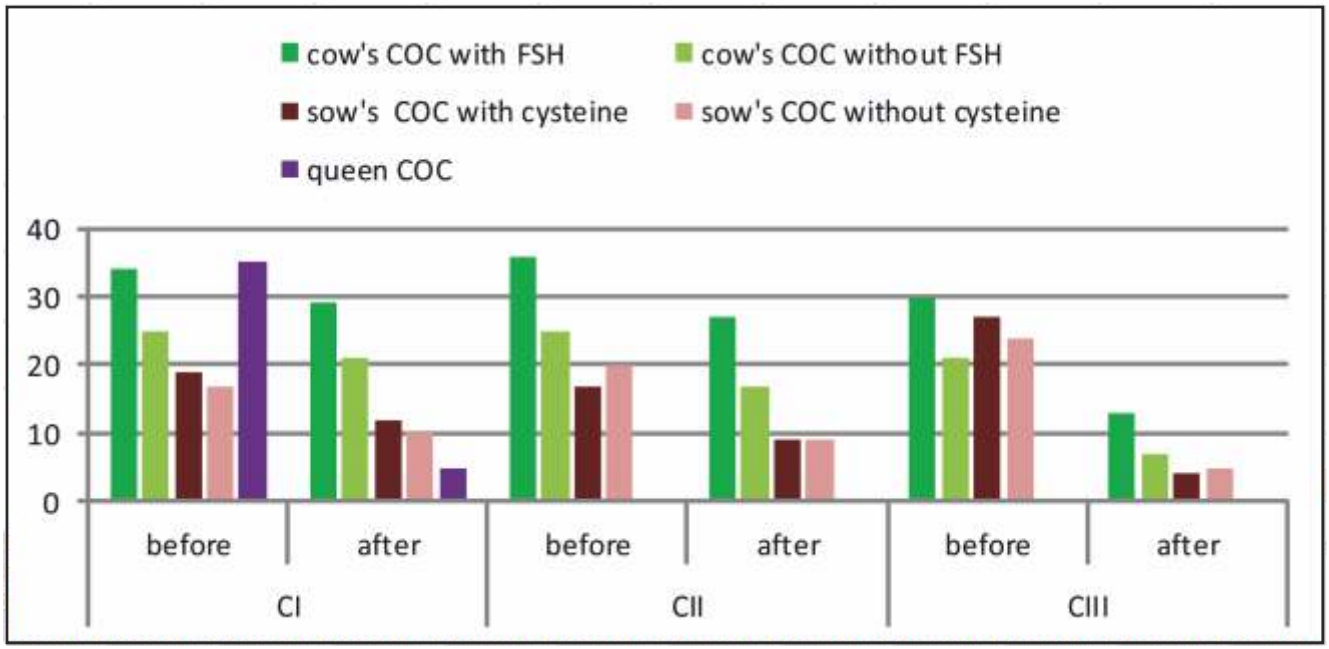

Fig. 1. Classification of cow, sow and queen cumulus-oocyte complexes (COC) based on their morphological aspects before and after *in vitro* maturation (IVM)

Their results suggest that LH is not needed *in vitro* because LH receptors are absent in oocytes and cumulus cells, they are expressed only in theca and granuloasa cells, and the meiotic induction effect of LH on CCs is thought to be indirectly mediated through the EGF-like receptors. FSH receptors are expressed in mural granuloasa cells and cumulus cells starting at the secondary follicular stage and can be concluded that in order for FSH to be effective *in vitro,* the size of follicules from which the COCs are harvested is important also [1].

Similar results were observed also in sow oocytes maturated in medium with cysteine, a precursor of the antioxidant glutation (GSH): in class CI 63,15% from COCs showed cumulus expansion, in class CII, 52.94% and 14.81% in class CIII and without cysteine: in class CI 58.82%, in class CII 45% and in class CIII 20.83%. Stable levels of GSH in IVM oocytes ensure a larger number of oocyte to be fertilized and reaching the blastocist stage. Glutathione is crucial for maintaining the redox state of cells and also protecting them against oxydative process [10].

In the same study, we analyzed expression of BCL-2 gene, a specific anti-apoptotic protein. The expression of BCL-2 was almost double in cumulus cells maturated in media with cysteine compared with cumulus cells cultivated in culture media without cysteine, suggesting that antioxidant supplementation can increase the viability of the oocyte [15].

In another study done by our group, we observed in bovine oocyte at 24h after culture that gene expression of BAX/BCL2 is subunitary, indicating their ability to adapt to their new environment [3].

In experiment 3, at 30 h after IVM, 14.28% from queen oocytes were with cumulus expanded. Examination of nuclear status after Hoechst 33342 staining revealed in oocytes cultured for 30h that 5.71 % of them were in GVBD and 2.85 % in M II, the rest of the oocytes examinated containing unidentifiable chromatin [12]. These results could be explained by the physiological status of the queens (2 out of 3 were pregnant). It is know that during pregnancy due to high steroidal hormones produce in fetal-placental unit and CL, follicle development may be retard [17].

Other results indicates that the reproductive cycle stage (inactive, follicular or luteal) of donor cat ovaries has no apparent effects on the frequencies of maturation and fertilization of oocytes, but influences developmental competence of the oocytes following IVM or IVF [7]. Compromised oocyte function during non-breeding season could be overridden by supplemental of *in vitro* culture media with FSH and antioxidant [4]. Several aspects of the first class COC used in the studies presented are shown in Figure 2.

In the fourth experiment, from total number of oocytes maturated in vitro (163 COC), 37 stared the division after fertilization (22.69%) of which 16 had 2 cells, 12 with 4 cells, 5 with 8 cells and 4 had reached blastocyst stage in the seven day after fertilization [6].

These results may be due to quality of COC used for IVF:there were more class III retrieved by puncture (32.62 COC / session) compare with CI and CII.

In vitro cultivation environment of embryos used can explain the low rate of blastocysts obtained from all cleavage cells (10.81%).

CONCLUSIONS

Bovine COCs quality, the size of follicles from which oocyte -cumulus are harvest and the culture media are important for *in vitro* nuclear maturation.

The maturation media supplemented with cysteine does not increase the number of maturated sow oocytes based on structural morphological aspects.

BCL-2 gene expression in sow cumulus cells registered a significant increase in COC cultivated in cysteine supplemented medium.

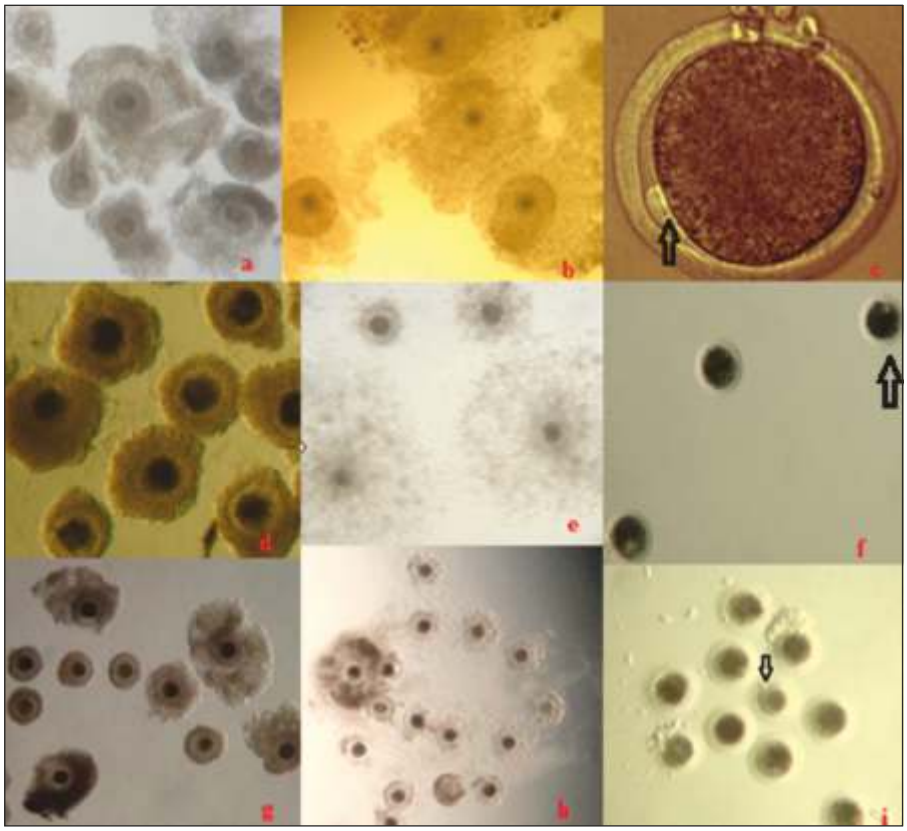

Fig. 2. First class COC from bovine specie (a. before IVM, 5X; b. after IVM, 5X; c. oocyte in MII, 40X →PBI), swine specie (d. before IVM, 5X; e. after IVM, 5X; c. denuded MII oocyte,3X) and cat specie (g. before IVM, e. after IVM, c. oocyte in MII, →PBI, 3X)

Reproductive stage (luteal phase) of the queen influence that IVM of oocytes; with only 2.85% of them in M II.

In vitro, culture media and environmental conditions in which bovine oocytes/embryos are kept can influence the results of this technique.

ACKNOWLEDGMENTS

The research was carried out in the *Assisted Reproduction, Cellular and Molecular Biology Laboratory* from "*Horia Cernescu Research Laboratories Complex*", equipped through POSCCE 2669 grant

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