

# AN OVERVIEW OF EMBRYO TRANSFER IN HORSES

## BACKGROUND

Embryo transfer refers to the procedure for collecting a fertilized oocyte (embryo) from a donor mare and transferring it to the synchronized reproductive tract of a recipient mare. Nowadays, the embryo is removed from the uterus of a donor mare and transferred to the uterus of a recipient mare; however, as technologies continue to develop, it may become feasible to collect oviductal embryos commercially for transfer to recipient mares.

The first embryo transfer (involving rabbits) was reported over a century ago; yet, this means of assisted reproduction was not developed for use in the horse until the early 1970's and did not become popular among horse enthusiasts until the last decade. Currently, most equine breed associations in the United States do permit embryo transfer. Notable exceptions include the Jockey Club (Thoroughbred breed), the United States Trotting Association and the American Miniature Horse Association. It is important to contact breed associations before implementing embryo transfer because of changing regulations regarding its acceptance, or specific policies regarding its use.

## BASIC TECHNIQUE

The customary technique for performing embryo transfer begins with synchronization of ovulation between a donor mare and the recipient mares, followed by insemination of the donor mare. The uterus of the donor mare is flushed 7 or 8 days after ovulation, and the retrieved embryo is transferred into the uterus of a recipient mare. This transfer can be accomplished either nonsurgically (via transcervical deposition of the embryo) or surgically (via laparotomy/uterotomy, laparoscopy-guided needle puncture, or transvaginal needle puncture). Under ideal conditions (i.e., use of fertile donor mares, recipient mares, stallions, and experienced personnel), one can expect an embryo recovery rate of 50-70% and an embryo transfer success rate of 50-70%, resulting in an overall pregnancy rate of 25-50% per cycle. If sub fertile donor/recipient mares or stallions are utilized, the ovulation between the donor mare and recipient mare is not well synchronized, or personnel are not experienced, the success rate will be reduced accordingly. This reduction in expected outcome can be quite dramatic and extremely disappointing.

## SELECTION OF DONOR MARES

There are numerous motives for commercial use of embryo transfer, including the following:

- *Management of sub fertile mares* - Some sub fertile mares may be good candidates for embryo transfer. For instance, mares, which become pregnant but repeatedly experience early embryonic death may benefit from embryo transfer as a therapeutic alternative. Mares with widespread endometrial periglandular fibrosis, cystic glandular distention, or endometrial cysts; mares with irreparable cervical disease; or mares with unexplained habitual abortion are good candidates for this procedure. The value of the donor mare's offspring should be the primary consideration in any decision to perform an embryo transfer. The procedure can be very expensive and is typically higher in sub fertile mares, because of sub optimal embryo recovery rates. Furthermore, embryo transfer is not the answer to restoration of fertility in all sub fertile mares. As evidenced by recent research, sub fertility in some older mares may be due to reduced intrinsic viability of oocyte, innate embryonic defects or oviductal dysfunction leading to interruption of early embryonic development. Embryo transfer would not correct

such factors.

- *Exploitation of superior mares* - Under usual circumstances, a mare is capable of producing a maximum of one foal per year, whereas a stallion may sire dozens to hundreds of foals per year. As a result, the stallion typically has much greater genetic influence on the breed than the dam. Although embryo transfer is unlikely to correct this imbalance, some breed associations will permit more than one foal to be registered per year from a dam, thereby increasing the mare's contribution to the gene pool. Even in those associations, which permit the birth of only one foal per dam per year, one may be able to facilitate pregnancies in mares earlier than otherwise advisable (e.g., at two years of age). Furthermore, it may be possible to register more than one embryo-transfer foal during the same calendar year if the mare is bred to stallions of other breed registries. For example, a Quarter Horse mare bred to a Paint stallion and is eligible for registration by the American Paint Horse Association, even if another embryo-transfer foal was obtained when the same mare was bred to an American Quarter Horse stallion and was registered by the American Quarter Horse Association the same year could produce an embryo-transfer foal. Embryo transfer may also be a viable option for use in mares, which give birth late in the breeding season. Embryo transfer offers the advantage of producing a pregnancy in these mares during the same year, yet allowing them to be rebred early in the following breeding season.
- *Reproductive management of performance mares* - Embryo transfer literally offers the option of allowing a mare to take a week out of competition to produce a foal. The mare is bred or artificially inseminated at the appropriate time, and then an embryo is collected at Day 7 or 8 post-ovulation for transfer to a synchronized recipient mare. The mare can then return to a performance career. Hence, the previous requirement of delayed training and competition to allow for production of a foal is eliminated.
- *Assessment of fertility* - Embryo transfer is occasionally used as a means to assess fertility of either mares or stallions. It can be especially useful to ascertain pregnancy rates of stallions whose semen has been processed prior to insemination of mares (e.g. to test whether the semen of a given stallion retains a high level of fertility when it is stored cool for 24 hours or is subjected to cryopreservation).

It is imperative that donor mares be in good condition for breeding. These mares should have normal reproductive cycles. If one is unsure of the fertility of a donor mare, a thorough breeding-soundness examination should be conducted in an attempt to detect any problems, which might affect the mare's reproductive performance. Before initiating embryo transfer with a given mare, the value of the mare and any offspring should be critically assessed. The costs associated with embryo transfer can be very high, particularly when intensive management of sub fertile mares is required.

## **SELECTION OF RECIPIENT MARES**

Recipient mares should be relatively young (i.e., 3-10 years of age), exhibit normal reproductive cyclicity and have no history of reproductive problems. A complete breeding-soundness examination should be conducted to ensure that recipient mares have no abnormalities, which would reduce their ability to carry a foal to term. Ideally, recipient mares should be at least as large or slightly larger than the donor mare. It is also important that the mares are easy to handle. If possible, mares with a history of good lactation and mothering ability should be chosen. Mares selected as recipients should be identified permanently (i.e., by freeze branding) to facilitate accurate record keeping.

## **SUPEROVULATION**

The hormonal preparations used to induce super ovulation in other animals are not successful in the horse. Injections of pregnant mare serum gonadotropin (PMSG or eCG) or porcine follicle stimulating hormone (FSH-P), or a combination of FSH-P and porcine luteinizing hormone (PLH) have yielded excellent results in other species, but decidedly poor results in mares. The reason that mares are non-responsive to PMSG probably stems from the fact that this hormone binds exclusively with LH receptors in the mare, whereas it has both FSH and LH activity in the other species. Although mares appear to be relatively refractory to FSH-P, repeated administration of high doses of this preparation can increase the ovulation rate slightly in mares. An increased incidence of double ovulations occurs, but this slight increase in ovulation rate probably does not justify the high cost of treatment.

Recent studies indicate that ovulation rate can be increased to an average of 3 per cycle in mares treated with crude equine pituitary extract, or an FSH-enriched fraction from the same extract.

Similarly, passive immunization of mares against the alpha subunit fragment of inhibin is reported to result in only a small increase in ovulation rate in mares (an average of 1.6 ovulations). This treatment regimen resulted in some undesirable side effects.

The inability to routinely super ovulate mares would create a major barrier to the success of embryo transfer programs in horses if the objective were to increase dramatically the number of offspring produced over the lifetime a mare. However, because of current breed association restrictions regarding the number of foals, which can be registered by embryo transfer per year from a given mare, the need to devise methods to super ovulate mares becomes less of a priority.

## **SYNCHRONIZATION OF OVULATION**

Considerable attention should be given to synchronizing ovulation between the donor and recipient mares to maximize the chance of success of the embryo transfer procedure. This feat is more difficult to accomplish in mares than in cows because of the greater individual variation in the length of estrus in mares and the effect of seasons on ovulation. Ideally, the recipient mare should ovulate from one day prior to two days after the donor.

If one has a large broodmare band from which to select mares in natural ovulatory synchrony with the donor mare, it is unnecessary to rely on hormonal protocols to induce synchronous ovulation. Prostaglandins and progestins have been used independently to attempt synchronization of ovulation in mares; however, neither preparation reliably induces the tight synchrony of ovulation that is required for embryo transfer. To help increase the likelihood of acceptable synchronization, the products should be administered to recipient mares one day after they are given to the donor mare. Human chorionic gonadotropin (hCG) can also be administered to mares to accelerate ovulation selectively where needed to improve the degree of synchrony between the donor and recipient mares. To help ensure that a well-synchronized recipient mare will be available at the time of embryo transfer, it is advisable to attempt synchronization of the donor mare with at least two recipient mares. This protocol also helps to ensure that sufficient recipient mares are available for embryo transfer, should twin embryos be recovered from the donor mare.

Prostaglandin are typically administered twice, 14 days apart, with the injections given to the recipient mares one day after the injections are given to the donor mare. Since prostaglandins only induce luteolysis when a mature, functional corpus luteum is present, it may be possible to administer only one injection each to the donor mare and recipient mares if all mares are confirmed to be in mid-

diestrus at the time of treatment. Preferably, mares receiving injections should have ovarian follicles of similar size at the time of treatment to help assure similar rate of follicular development following prostaglandin injection.

To synchronize ovulation, progestins can be given to mares to lengthen the luteal phase of the cycle artificially. The progestins are typically administered to all mares for 14-15 days, with prostaglandins being administered concurrently on the last day of progestin treatment to regress any retained corpora lutea. The orally active progestin, altrenogest (Regumate®) is oftentimes used for regulating synchronization of ovulation. A drawback of progestin treatment is that ovarian follicles of mares are not uniformly regulated, resulting in varying intervals from termination of treatment to ovulation.

Synchronization of ovulation is most effectively accomplished using a combination of the hormones progesterone and estradiol-17 $\beta$ , because this treatment inhibits follicular development more uniformly than does progesterone alone. The result is less variation in timing of ovulations. Progesterone (150 mg) and estradiol-17 $\beta$  (10 mg) in oil are administered daily for 10 days, with prostaglandin given on the last day of steroid treatment. If HCG is administered when a 35-mm follicle is detected, approximately 70-75% of mares ovulate on Days 10-12 following the termination of treatment. A single injection of a microsphere preparation, designed to deliver 1.25 gm progesterone and 100 mg estradiol-17 $\beta$  at a controlled rate for duration of 12-14 days, was recently tested for efficacy of ovulation synchronization. When prostaglandin was given 14 days following the steroid injection, variation in number of days to ovulation was reduced significantly when compared to a similar microsphere injection containing only progesterone. Incorporation of estradiol-17 $\beta$  in the treatment regimen also seemed to promote better expression of post-treatment estrous behavior. Progesterone-estradiol preparations are not yet approved federally for use in horses.

If a recipient mare ovulates more than 3 days after the donor mare, it is advisable to administer progesterone in oil (300 mg per day) to the recipient mare, beginning on the day on which ovulation occurs. This treatment may result in a suitable embryo-transfer recipient, even if the mare ovulated 5-6 days after the donor mare. The recipient mare should be kept on the progesterone supplementation for at least 5 days, until the mare's corpus luteum has become fully functional. This treatment is ineffective if the recipient mare ovulates far in advance (> 1-2 days) of the donor mare.

One way to avoid the difficulties associated with synchronizing ovulation in mares is to use ovariectomized, progesterone-treated mares as embryo recipients. Progesterone in oil (300 mg) is administered intramuscularly to recipient mares 2 days after the donor mare has ovulated. Thus, the recipient mares are progesterone-primed for 5-6 days prior to transfer of the embryo. If an embryo transfer is performed, the progesterone treatment is continued until Day 100-150 of gestation. Thereafter, placental progestin production is sufficient to maintain the pregnancy to term. If an embryo is not transferred, or a pregnancy is not detected by transrectal ultrasonography at Day 12-15 of gestation, the progesterone is discontinued. The progesterone should not be administered for at least one week before reinitiating supplementation for a subsequent transfer. Ovariectomized mares have normal gestational length, as well as normal parturition, lactation, mothering ability and post-partum uterine involution, and can be used again as embryo transfer recipients. Ovariectomized mares should probably be used within 6-12 months after the surgery is performed to avoid extensive atrophy of the endometrial tissue.

Some workers have used Altrenogest successfully as the sole progestin supplement in ovariectomized recipient mares. One study suggests that this product may be inferior to progesterone in oil during the initial stage of pregnancy; however, it one can switch from progesterone supplementation to altrenogest by approximately Day 35-40 of gestation.

## EMBRYO RECOVERY

A non-surgical transcervical procedure is used to procure the embryo from the uterine lumen of the donor mare. This is usually attempted on Day 7 or Day 8 post-ovulation. Embryo recovery rate are lower when attempted at earlier times, and embryos are too large to manipulate without damage when recoveries are attempted later than Day 8. Embryos are easier to identify on Day 8 because of their larger size, but recovery on Day 7 is considered preferable by some workers because the embryos are smaller and, therefore, less susceptible to trauma induced during the collection and transfer procedures.

To prepare for an embryo recovery procedure, the donor mare is first placed in a set of stocks, and her tail is wrapped and elevated. Manure is removed from the rectum, and then the hindquarters are prepared aseptically, as one would do prior to entering the vagina to obtain a uterine swab for culture. It is important to rinse all providine-iodine scrub from the hindquarters (including the vulvar opening and vestibular lumen) before continuing with the embryo recovery, because the iodine is embryo toxic.

For embryo recovery, a uterine flushing catheter and sterile examination sleeve are required. We recommend a 75- to 120-cm long balloon-tipped silicone catheter (28-33 Fr) for embryo retrieval. Using a sterile plastic sleeve to cover the hand and arm of the operator, the catheter tip is delicately passed through the cervical opening and into the posterior uterine body. A small amount of sterile, nontoxic water-soluble lubricant should be applied to the outside of the sleeve and catheter prior to attempting placement of the catheter in the uterus. The balloon cuff of the catheter is then filled with 50-70 cc of air or water, and pulled posteriorly to establish a good seal at the junction of the uterine body and internal cervical os.

Modified Dulbecco's phosphate buffered saline (D-PBS) containing 1% (v/v) neonatal calf serum (NCS) or fetal calf serum (FCS) and penicillin-Streptomycin is generally considered to be the medium of choice for the uterine flush. Volumes of 1000-1500 ml (30-37°C) are typically used for each flush and the procedure is repeated three times. Tubing to the uterine catheter connects the bottle containing the flush solution, and the fluid is allowed to enter the uterine lumen by gravity flow. The fluid should be retained in the uterine lumen for three minutes before retrieving it. Manipulation of the uterus *per rectum* during fluid infusion into the uterus will permit more uniform filling of the uterine lumen. The fluid is either collected in 1000-ml graduated cylinders, or allowed to pass through specially designed (70-75µm pore size) embryo filters to permit escape of fluid and retention of any collected embryos. If the filter is used, it is important to make certain that the filter device always contains a small amount of fluid, so that any collected embryos are continually bathed in the fluid and not subjected to desiccation. At least 90% of the infused fluid should be retrieved; if not, the mare can be given 20 I.U. oxytocin intravenously to aid evacuation of the fluid within the uterus. Additionally, the uterus can be manipulated *per rectum* to help remove retained uterine fluid. If a filter was not used in-line to collect embryos, the retrieved fluid in the graduated cylinders should be allowed to stand undisturbed for 10-15 minutes, thereby allowing time for any contained embryos to sink to the bottom of the cylinder. Then, all but 50 cc of fluid is siphoned carefully from the top of each cylinder. All siphoned fluid is passed through an embryo filter, in case any embryos are accidentally siphoned with the fluid.

The recovered fluid is transferred to a sterile round Petri dish (typically 100- to 150-mm diameter and 15-mm depth) with a cover. The filter assembly should be rinsed several times to ensure that the embryo is rinsed away from the mesh. The fluid in the Petri dishes is examined using a dissecting microscope (7x to 25x magnification) to identify any embryos. The contents of the dish can be gently swirled prior to examination in an effort to move the embryo to the center dish.

Identified embryos are aspirated into a sterile 0.25- or 0.5 cc French straw and transferred into a small 4- to 6-well embryo wash dishes containing filter-sterilized flush medium with 10% (v/v) NCS or FCS. The embryo is washed 4-6 times by transferring it into the various wells of the wash dish. It is then ready for transfer into a recipient mare. It can be stored in the covered wash plate for 3-6 hours at room temperature (20-25°C) prior to transfer.

The D-PBS, NCS, and FCS can be purchased commercially. The DPBS can also be prepared in the laboratory, but purchase of the products ensures proper measurements of solutes and ideal water quality. The products can be purchased from basic science laboratories or embryo transfer supply companies. The embryo transfer supply companies can also provide catheters, tubing, filters, and Petri dishes for embryo identification.

## **EMBRYO TRANSFER**

Embryos can be transferred either nonsurgically or surgically into a synchronized recipient mare. The nonsurgical approach involves transcervical deposition of the embryo into the uterine body. To perform this procedure, prepare the hindquarters of the recipient mare as described above. Load the embryo into either: 1) a 21- to 25-inch insemination pipette, or 2) a 0.25- or 0.5-cc capacity straw for frozen semen. If the embryo is loaded into the insemination pipette, a 10- to 12-cc syringe containing approximately 5-6 cc of air should first be secured to the appropriate end of the pipette, then the medium and embryo should be loaded into the syringe in 0.5-ml (1-inch) increments using the following sequence: medium, air, medium, air, medium containing embryo, air, medium, air. This loading procedure minimizes movement of the embryo within the pipette and helps assure that it will be discharged into the uterus at the appropriate time. We generally recommend using a 0.25- to 0.5-cc French straw for embryo transfer to reduce the volume of fluid entering the uterus of the recipient mare. The straw is loaded in a manner similar to that described for the insemination pipette, except that the volume is reduced significantly. The contents are transferred to the recipient mare using a commercially available insemination gun developed for use with French straws. If a 0.25- or 0.5-ml French straw is used to load the embryo, flexible tubing attached to a 3- to 6-ml syringe is first fitted to the end of the straw, which contains a plug of cotton and polyvinyl chloride (PVC) powder. Load the embryo and medium into the straw by aspiration with the syringe. Wetting the PVC powder creates a seal on the end of the straw attached to the tubing and syringe.

Using a sterile lubricated sleeve to cover the hand and arm of the operator, place the tip of the loaded pipette or insemination gun in a cupped palm, and carefully guide the tip of the pipette along the vagina to the external cervical os. Use the index finger to dilate gently the external cervical os of the recipient mare, then digitally surround and compress the circumference of the external cervical os, while passing the tip of the pipette through the cervical lumen into the uterine body. Once resistance is met with the pipette, it is pulled posteriorly approximately 1-2 cm and the embryo and medium are expressed slowly from the pipette into the uterine body, using the air pre-loaded in the syringe lumen. Some workers place the insemination pipette in a guarded sheath prior to embryo transfer to reduce contamination of the embryo during the transfer process. We recommend that one hand be placed in the rectum to gently elevate the uterus so that the insemination pipette can be advanced to the bifurcation of the uterus without creating irritation to the endometrial surface; then the embryo is discharged into the uterine lumen.

Studies to date suggest that administration of antibiotics or anti-inflammatory drugs to the recipient mare at the time of transfer are of no benefit. Exogenous progestins are also of questionable

value, unless the recipient mare requires supplementation because of ovariectomy or asynchronous ovulation, as described above.

Some laboratories prefer surgical transfer of embryos. The standard surgical embryo transfer is now performed via flank laparotomy. Place the mare in a set of stocks and give her detomidine hydrochloride (10-20 mcg per kg body weight) for sedation. Surgically clip and scrub a rectangular area (extending from the last rib to the tuber coxae and from the transverse processes of the lumbar vertebrae to a point 40 cm ventrally), then drape the area for aseptic surgery. Perform a vertical line block (skin and underlying tissue), using 2% lidocaine hydrochloride (~50 ml), from a point 5 cm beneath the transverse processes of the lumbar vertebrae and extending 25 cm ventrally, midway between the tuber coxae and last rib. A 15-20 cm vertical skin incision is made over the line block, beginning 10 cm beneath the transverse processes of the lumbar vertebrae. Dissect bluntly the underlying musculature, and incise the peritoneum to access the peritoneal cavity. Place a hand in the peritoneal cavity to grasp the nearest uterine horn, and exteriorize its proximal portion through the flank incision. Use a cutting needle tip of a sterile paper clip to puncture the uterine wall at an a vascular location, then place the embryo in the uterine lumen via the puncture site, using a 0.25-mm French catheter. Compress digitally the puncture site in the uterine wall for one minute, and then replace the exposed uterine horn in the peritoneal cavity. Appose the peritoneum, muscular layers, subcutaneous tissue, and skin along the incision site with suture material in routine fashion. Return the recipient mare to a stall for 5-7 days to monitor her surgical site and general health. Systemic antibiotics are generally administered for 5-7 days, but exogenous progesterone or anti-inflammatory drugs are usually not considered to be necessary. Return the mare to a paddock or pasture in 5-7 days if no post-surgical complications arise.

Recently, two additional techniques have been described for surgical transfer of embryos. One approach involves transfer of the embryo to the lumen of a uterine horn by pulling the uterine horn posteriorly to position it against the anterior vaginal wall with one hand in the rectum. Then, a needle placed in the vaginal vault is used to perforate the vaginal and uterine walls, and a Teflon tube is guided through the lumen of the needle to place an embryo in the uterine lumen. Another approach utilizes an endoscope/laparoscope placed through the flank wall, with needle puncture and embryo placement in the uterine lumen, as described above. More work is needed with these procedures before they can be recommended over the standard surgical approach.

The advantages of the non-surgical technique over the surgical technique are simplicity, reduced expense, and reduced post-transfer complications. Pregnancy rate achieved with the surgical approach is reported to be higher by some laboratories; however, some workers have reported pregnancy rates following non-surgical transfer to be similar to those achieved with surgical transfer. Technician variation probably contributes greatly to the differences noted among laboratories regarding pregnancy rates following non-surgical embryo transfer.

## **STORAGE OF EMBRYOS**

Cryopreservation of equine embryos has met very limited success. Equine embryos seem to tolerate the freezing/thawing process when in a morula/early blastocyst stage and < 200µm in diameter. Unfortunately, embryos recovered at Day 7 or 8 of gestation are 400-1200µm in diameter. Embryos recovered at Day 5-6 are in the acceptable size range for freezing; however, embryo recovery rates in the uterus are extremely low on Day 5, and are lower on Day 6 than on Day 7 or 8. This lowered recovery rate is observed because the embryo does not typically enter the uterus until Day 6 post ovulation. Development of techniques to accelerate oviductal transit of embryos, or to access the oviductal lumen to retrieve embryos, may increase the commercial feasibility of embryo

cryopreservation.

Storage of embryos for 12-24 hours at refrigerated temperatures prior to transfer is becoming increasingly popular. This process permits transport of embryos to distant locations for transfer to recipient mares. A container especially designed for cooling and transporting equine semen, the Equine Express II cooled semen & embryo transport system can be used for transporting equine embryos. Ham's F10 media containing 10% NCS or FCS and gassed with a mixture of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> appears to provide optimal conditions for transport of equine embryos. Embryos and media can be loaded into small-capped tubes prior to transport. Refer to the attached protocol for transporting cooled embryos for transfer.

## **Materials and Protocol for Cooled Transport of Equine Embryos**

### **Materials**

- Vigro Flush Media
- Vigro Holding Media
- Sterile Pasteur pipette
- 5-ml snap-cap vials (sterile)
- 50-ml plastic conical-bottom centrifuge tubes (sterile)
- Parafilm sealing film
- Equine Express II cooled semen & embryo transport system

### **Protocol**

1. Place approximately 1mL Vigro Holding Media in a 2-ml Nalgene Tube.
2. Transfer the embryo to the 2-ml vial, using a 0.5-ml straw, allow to settle for 2 minutes
3. Top off the tube drop by drop until the tube is crowned and carefully screw the cap in place.
4. Secure well with Parafilm
5. To ensure successful transfer of the embryo to the vial, transfer the embryo on the dissection microscope with appropriate back lighting. Verify that the embryo is in the 2-ml vial before proceeding with the storage/transport steps,
6. Place approximately 45 mL of Vigro Flush Media in the 50-ml centrifuge tube, then gently place the filled 5-ml vial containing the embryo in this tube and fill it to the top with additional media.
7. Secure the cap tightly, then secure well with Parafilm.
8. Load the filled 50-ml centrifuge tube into the body of the Equine Express II cooled semen & embryo transport system. Place the Thermoregulator on top of the centrifuge tube and lay the Equine Express II Special Coolant Pac on top of the plate. Next place the lid down on to the body of the Equine Express II
9. Place appropriate paper work above the lid in a plastic bag. This paper work should contain information regarding the mare, stallion, owner, veterinarian, embryo quality, and age, and date and time of embryo collection.
10. Lower the corrugated lid into the side slots, slide the plastic tamper proof tie in place and send to the intended recipient by overnight carrier or counter-to-counter airline service.

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