Relative expression levels of selected target genes in dairy cows produced by artificial insemination or multiple ovulation embryo transfer

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SUMMARY

Adoption of superovulation for in vivo production of cattle embryos has been one of the most successful reproductive biotechnologies among cattle breeders in order to produce male and female offspring of superior genetics. However, the effect of this technique on the performance of the resulting offspring is still unclear in terms of health, productivity and fertility. In this study, the primary objective was to evaluate the effect of artificial insemination (AI) and MOET heifer production protocols on the relative expression levels of 3 fertility-related genes (SAXO2, TAC3, and TFF2) in dairy cattle. This experiment was carried out on a total of ten primiparous Holstein cows belonging to a private dairy herd in north-western Egypt. Cows were divided into two groups (MOET-cows, n=5) and (AI-cows, n=5). MOET-cows were produced by superovulation and embryo transfer, while AI-cows were produced by AI using conventional semen from approved sires. Both, MOET-cows and AI-cows were submitted to the presynch-ovsynch protocol on day 45 after their first calving and were timely inseminated on day 80 postpartum. Cows received two Prostaglandin f2 alpha injections (500 Ug cloprostenol sodium) on days 45 and 59 postpartum, then ovsynch protocol was applied on day 70 (first GnRH, 12 Ug busrelin), 77 (500 Ug cloprostenol sodium), 79 (12 Ug busrelin). Blood samples were obtained from individual cows on the day of timed artificial insemination (TAI) in the two groups. Relative expression levels of the target genes were determined by using qRT-PCR. Results showed significant associations between MOET heifer production technique and rising of relative expression levels of selected target genes (SAXO2, TAC3, and TFF2), where levels of the three genes were significantly greater in MOET-cows, compared to AI-cows suggesting upregulation of such genes in cows produced by MOET technique and may provide a partial explanation of the greater fertility observed in MOET-group.

KEY WORDS

MOET; TFF2; SAXO2; TAC3; Genes.

INTRODUCTION

Dairy cattle can be used as a model to study the genetic basis of fertility in mammals. Fertility traits are continuously recorded in large numbers, and whole genome sequence data are available to examine the major relatives of modern dairy cattle populations **(1).** Fertilizing several oocytes in a shorter amount of time to create higher-quality embryos that are then transferred to the recipient and raise the birth rate is the foremost definition of multiple ovulation and embryo transfer technology (MOET) **(2).** MOET is crucial to the worldwide flow of genetic resources in addition to protecting premium dairy herds, lowering the danger of exotic diseases and production expenses, and eliminating transportation stress **(3).** Although there are fewer ET breeding programs than AI breeding, AI breeding is still widely used worldwide **(4).** It has been cited that AI has been widely used in farm animals and is acknowledged as the best method for boosting reproductive potential **(5).**

A gene is a DNA sequence that is described as one or more sequences connected to RNAs and proteins **(6).** According to the Human Genome Nomenclature Organization, a gene is a bit of DNA that affects phenotypic or function **(7).** Since housekeeping genes are found in almost every cell in an organism, they are usually considered essential genes required for the preservation of basic cellular functions **(8).** Since reverse transcription-polymerase chain reaction (RT-PCR) is widely employed to measure mRNA levels, the β-actin housekeeping gene is often utilized as a denominator for sample comparisons **(9).** Multiple studies have been carried out in order to probe into specific genes that indicate cattle fertility. **(10)** Found 3 genes (SAXO2, TAC3, and TFF2) that were differentially elevated in AI-pregnant heifers compared to non-pregnant ones, they also mentioned that the transcriptome profile in the peripheral white blood cells (PWBC) of heifers at insemination predicted the heifer's fertility potential. In comparison to heifers that became pregnant through AI, research revealed that non-pregnant heifers had lower transcript abundance for SAXO2 in their (PWBC) and higher transcript abundance for the genes (TAC3 and TFF2) **(10).** In addition, the transcript levels of TAC3, TFF2, and SAXO2 were higher in primiparous MOET-heifers than in primiparous AI-cattle **(11).** The primary objective

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of the present study was to determine the relative levels of expression of three fertility-linked genes in blood samples obtained from primiparous cows produced by MOET or AI.

MATERIALS AND METHODS

Animals, housing and management

The procedures of the current work were approved by the local animal care and use committee, Beni-Suef University, Egypt **(BSU-IACUC: 022-256).** This study was carried out on ten primiparous Holstein cows belonging to a private dairy herd in north-western Egypt. Animals were >60 days postpartum and were selected in good body condition score (3.5) and had an average daily milk production of 36.52±3.23 Kg. All cows were checked by trans-rectal ultrasonography before enrollment to the experiment and were free from any health or reproductive problems during the entire period of the experiment. Cows in this experiment were kept in the same group due to similar ages and similar levels of milk production after their first calving. Cows in both groups (MOET-cows and AI-cows) were milked thrice daily at 6 am, 2 PM and 10 PM using and automatic milking parlor (Avikim). The food was distributed at milking times where cows returning from the milking parlor find freshly deposited food and still stands for a while. This will ensure closure of the teat sphincter before a cow is recumbent as a mastitis control strategy. A strict herd health program was applied in the herd. Parturient cows were cautiously monitored during calving and any dystocia case was promptly manipulated. Then cows were monitored during the first 15 weeks postpartum for probable health or metabolic problems and affected cows were treated accordingly.

Heifer production protocol

This experiment included five primiparous cows that were born as heifers produced through transfer of embryos obtained by multiple ovulation embryo transfer procedures applied on the farm (MOET-cows, n=5), and another five primiparous cows produced by artificial insemination (AI-cows, n=5).
The standard procedures of superovulation and embryo transfer are described briefly as following:

Superovulation

The protocol of superovulation was simply that applied by **(12)** as shown in figure (1).
Individual embryo donor cows received controlled internal drug release inset (CIDR; 1.39 g of Progesterone; Pfizer Animal Health, New York, USA) on Day zero. All animals received 9 mg of gonadotropin releasing hormone agonist (IM; Buserelin, Receptal, MSD Animal Health, New Cairo, Egypt) on Day 2. Ovarian superstimulation protocol for all animals included twice daily intramuscular (IM) injections of decreasing doses of porcine follicle stimulating hormone (FSH; Folltropin V, Bioniche Pharma, LLC, Lake Forest, Illinois, USA) for four days, starting with 80 mg on Day 4, for a total dose of 400 mg. On Day 7, all animals received 500 mg of Cloprostenol (IM; Estrumate, MSD Animal Health, New Cairo, Egypt) Day 4 (Day 7 of FSH treatment) by IM route and CIDR insert were removed. Animals were given a second dose of Cloprostenol and Buserelin one day later. The cows were observed twice daily for estrus expression. Donor cows were artificially inseminated 2 times with frozen semen 48 and 60 h after CIDR removal. Embryos were collected on day seven post-insemination.

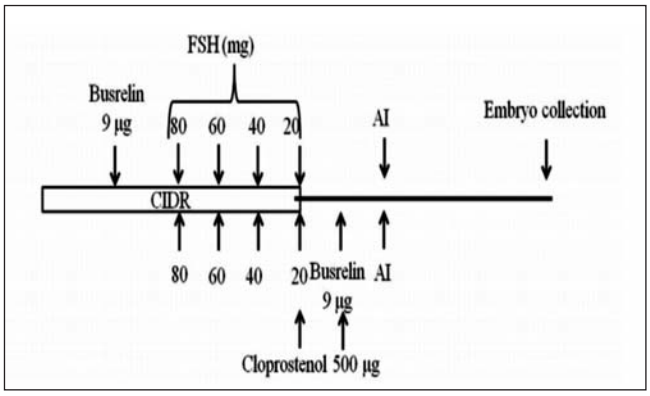

Figure 1 - Schematic presentation of the superovulation protocol (12).

Embryo flushing

Embryos were recovered non-surgically using a two-way foley catheter on day seven after insemination of embryo donors. The flushing process involved pumping 250 ml of VIGRO flushing medium into each uterine horn and each horn was flushed separately on several occasions (40-50 ml per occasion). The donor was restratined in a suitable ET working chute, administered with 4-7 ml xylocian HCl 2% solution to induce posterior epidural anaesthesia **(13).**
After preparation of the flushing set, the stainless steel stylet was inserted inside the two-way foley catheter and was fitted using a suitable forceps. The vulva and the perineal region were scrubbed using soap and water, dried and sprayed with iodine 10% than washed and dried again using clean towels. The foley catheter was inserted into the cervix as forward to the base of the middle third of the uterine horn to be flushed and the stylet was removed. Then a suitable amount of air (12-18 cm^3) inflated the balloon of the foley catheter to maintain it in its position and to prevent escape of the flushing fluid into the cervix. Using the Y-junction, a controlled flow of 50 ml of the flushing medium was allowed to fill the horn to be flushed, several massages were applied to the horn and the flushing medium was allowed to flow down through the other arm of the Y-junction into an embryo filter. The process was repeated several times till 250 ml of the flushing medium was used. Then the air in the foley catheter balloon was sucked to empty the inflation and the foley catheter was removed and the entire process was repeated with the second horn.

Embryo grading

The embryo evaluation criteria followed the criteria pertaining to first grade embryos as described by the international embryo transfer society **(14).** The embryo was symmetrical with suitable size and homogenous blastomeres occupying more than 85% of the embryonic mass. Minor blastomer extrusions or degenerations were reported upon evaluation under the stereo microscope. Figure (2) represents an image of first grade embryos.

Embryo transfer

Recipient cows were checked on day 6 after estrus to evaluate the quality of the corpus luteum and to determine the side at which the transfer process would be applied using trans-rectal ultrasonography (Sonoscape E1 Vet, probed by a multiple

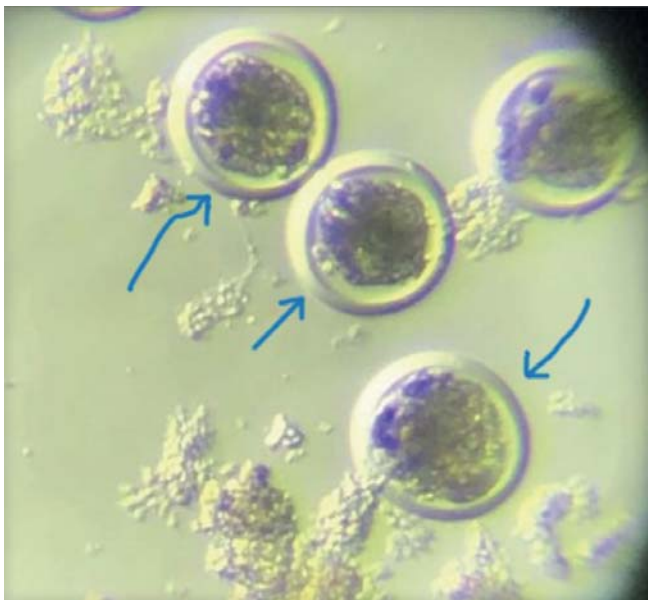

Figure 2 - First grade bovine embryo (Morula" upper left arrow" Compact morula " middle arrow" blastocyst "lower right arrow") obtained from donor cows.

frequency linear trans-rectal probe).

On day 7, after flushing the donor and evaluation of the embryos, first grade fresh embryos were loaded into 0.25 ml straw using an insulin needle. The loading process was carried out using the flushing medium. Firstly a suitable amount of the medium was aspirated into the straw, then an equal amount of air followed by a suitable amount of the medium containing the embryo, then equal amount of air and another amount of the medium. The straw was then loaded into the ET gun and covered with the sheath. The recipient was restrained in a chute and administered with 4-7 ml xylocaine HCl 2% solution in the sacro-coocygeal articulation to induce epidural anaesthesia. The vulva was rinsed washed and cleaned then dried with towels. The hand in the rectum guided the ET gun through the upper third of the uterine horn ipsilateral to the ovulated ovary and embryo was deposited **(11).**

The other group of heifers (AI-cows, n=5) was produced by artificial insemination using frozen-thawed semen from fertile sires.

Reproductive program

On day 45 postpartum individual cows were submitted to a standard presynch-ovsynch program for timed artificial insemination (TAI). The program began with two PGF2 alpha injections with 14 days interval (day 45 and day 59 postpartum). Twelve days later, i.e. on day 70 postpartum, the ordinary ovsynch program was applied and started with IM injection of 12 micro-gram busrelin (3 ml receptal, MSD, Egypt) followed by IM of 750 micro-gram cloprostenol sodium (2.5 ml estrumate, MSD, Egypt) seven days later, i.e. on day 77 postpartum and a final dose of busrelin (2.5 ml receptal, MSD, Egypt) on day 79 and cows received timed insemination on day 80 postpartum using frozen-thawed semen from approved sires. It should be noticed that cows which display estrus after any of the scheduled hormonal injections of the program were removed from the program and were checked and inseminated after estrus confirmation by experienced farm veterinarians.

Blood sampling

On the day of (TAI), blood samples were obtained from primiparous cows in AI- cows and MOET-cows groups. Vaccutainer tubes with EDTA were used to withdraw a 5-ml blood from the tail vein of each cow and samples were delivered to the molecular biochemistry lab. Faculty of Veterinary Medicine, Cairo University for gene analysis.

Determination of the relative expression levels of the target genes using qRT-PCR

The RNeasy Mini Kit (Qiagen Cat No. /ID: 74104) was used to isolate the T- RNA from fresh blood samples according to the manufacturer's guidelines. Both the purity and concentration of the isolated RNAs were assessed using the Nanodrop technology. The First-strand cDNA synthesis was performed using Superscript reverse transcriptase (Thermoscientific) using oligo DT then QRT-PCR was employed to assess the transcript level of the target genes using SYBR ™ Green PCR Master Mix (Thermoscientific Cat #: 4309155). The primer sequences were shown in table 1 and the QRT-PCR was performed as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds; 60°C for 30 seconds. Each assay was performed three times. The ACTB gene was used as an internal control to calculate the relative expression level. The relative expression data were calculated using CT, ΔCT, ΔΔCT, and 2- ΔΔCT conditions.

ΔCt = Ct (gene of interest) - Ct (housekeeping gene)

ΔΔCt = ΔCt (treated sample) - ΔCt (control average)

Fold gene expression = 2^ -(ΔΔCt)

Statistical analyses

Data were statistically analyzed using SPSS software volume 22. The relative expression levels of the three genes (TAC3, TFF2 and SAXO2) were compared between AI-cows and MOET-cows.

Table 1 - Primers sequences used for quantitative real-time PCR.

Gene symbol	Gene description	Accession number	Primer Sequence
TAC3	Tachykinin precursor 3	NM_181017.2	F: 5′-ACATCGTGAAGACACCCACC-3′ R: 5′-TCCAGGGCCATCTGTAGTCA-3′
TFF2	Trefoil factor 2	NM_001083521.1	F: 5′-TTTCAATCCCCTCCCGAAGC-3′ R: 5′-AAACCCAGCAACTGGGGAAA-3′
SAXO2	Stabilizer of axonemal microtubules 2	XM_005221895.4	F: 5′-CAGCCACCTCGACTATGTCC-3′ R: 5′-ATGGGGCACGTAATGAGACC-3′
ACTB	Actin Beta	NM_173979.3	F:5'- GCAGGAGTACGATGAGTCCG-3' R: 5'- TGTCACCTTCACCGTTCCAG -3'

Significance was set at P<0.05. ACTB gene was used as an internal control to calculate the relative expression level. The relative expression data were calculated using CT, ΔCT, ΔΔCT, and 2 - ΔΔCT conditions.
ΔCt = Ct (gene of interest) - Ct (housekeeping gene)
ΔΔCt = ΔCt (treated sample) - ΔCt (control average)
Fold gene expression = 2^ - (ΔΔCt)

RESULTS

Figures 3, 4 and 5 display the relative expression levels of the three investigated target fertility-linked genes TAC3, TFF2 and SAXO2 in AI-cows and MOET-cows.
Findings revealed a significantly (P<0.05) greater levels of TAC3 in MOET-cows (2.5 fold change), when compared to AI-cows (1 fold change) at timed insemination.
Similarly, MOET-cows displayed significantly (P<0.05) greater levels of TFF2 in PWBC obtained from MOET-cows at timed AI, as compared to AI-cows (10.1 fold change, compared to 1 fold change, respectively).
The same results were observed for SAXO2 levels in PWBC from MOET-cows at timed insemination (2.1 fold change), compared to 1 fold change in AI-cows.

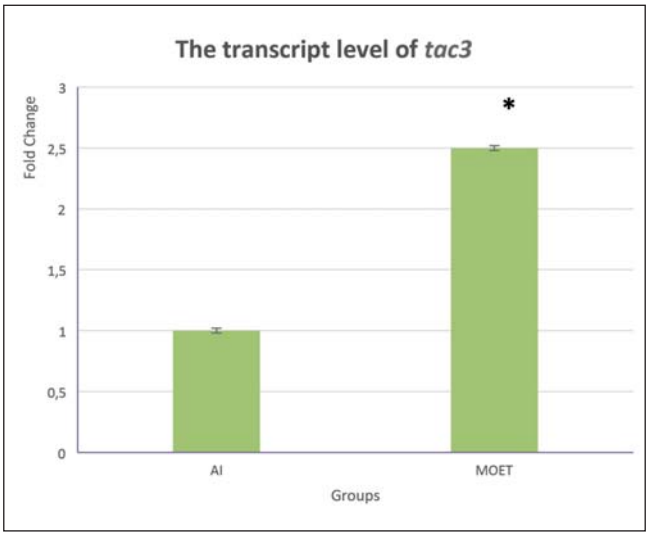

Figure 3 - Relative expression levels of TAC3 in PWBC from AI-cows and MOET-cows at timed insemination (P<0.05).

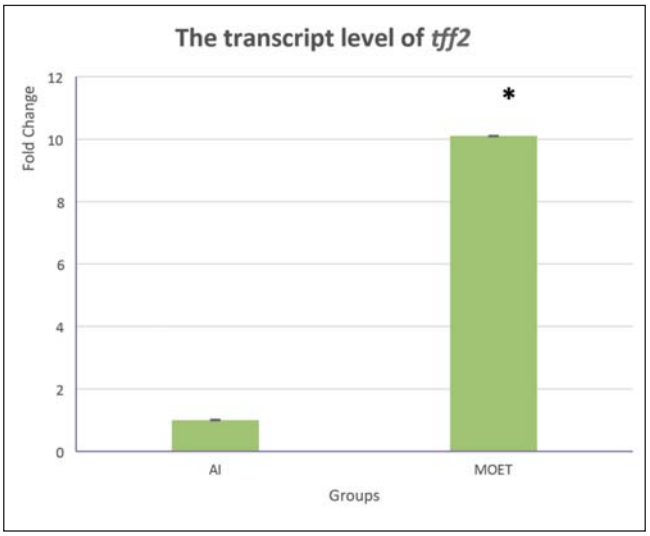

Figure 4 - Relative expression levels of tff2 in PWBC of MOET-cows and AI-cows at timed insemination (P<0.05).

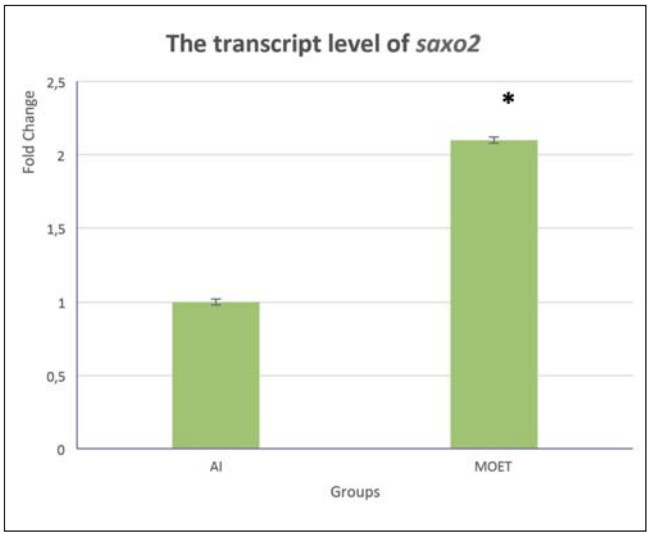

Figure 5 - Relative expression levels of SAXO2 in PWBC at timed insemination in AI-cows and MOET-cows (P<0.05).

DISCUSSION

The results showed that MOET heifers showed higher expression of genes associated with fertility and performance (TAC3, TFF2 and SAXO2) compared to AI heifers, suggesting a potential role for assisted reproductive technologies in improving genetic performance.
This study is consistent with the results of **(11)** which revealed that the transcript levels of TAC3, TFF2, and SAXO2 were higher in primiparous MOET-heifers than in primiparous AI-cattle. These results suggest that production and fertility rates in dairy cows can be improved through the use of embryo transfer techniques, which may lead to increased production efficiency in agricultural sectors.
Our results coincide with that reported by **(11),** which revealed that MOET-heifers had higher levels of the examined fertility marker genes (TAC3, TFF2, and SAXO2) in their blood samples than AI-heifers. The neurokinin B protein, which is coded by the TAC3 gene, promotes the production of gonadotropin-releasing hormone (GnRH), that is essential for the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) **(10).** The Trefoil Factor 2 gene can be reflected into the constant secretory protein TFF2, considering the Trefoil Factor 2 gene protects the reproductive system's epithelial cells, it may increase fertility **(11).** Moreover, it maintains the mucus layer, shields the mucosa from damage, and influences epithelial recovery **(15).** The gene for Stabilizer of Axonemal Microtubules 2 (SAXO2) maintains the cytoskeleton that makes up a cilium or flagellum's core further many cells, organisms, and microorganisms have flagella and cilia to aid in motility and bending **(11).** On the other hand, **(16)** cited that heifers that become pregnant following AI have been shown to have elevated SAXO2.
In conclusion, our study shows the positive influence of MOET protocol used for heifer production on relative ex-

pression levels of selected target genes (TAC3, TFF2, and SAXO2) related to dairy cattle fertility.

CONCLUSION

Collectively, our findings confirmed previous research results that show MOET heifers showed higher expression of genes associated with fertility and performance (TAC3, TFF2 and SAXO2) compared to AI heifers.

Ethical approval and consent to participate

All experimental work on live animals was approved by the Institutional Animal Care and Use Committee, Beni-Suef University, Egypt (BSU-IACUC: 022-256).

Acknowledgement

Thanks are due to all the authors in charge of the research who gave their utmost effort in completing this work, and thanks are due to the head of the department under whose supervision this work was carried out.

Author Contributions

Shrouk Abaas: participated in writing the research and is considered the corresponding author for submission the research and contributed to analyzing the samples by qRT-PCR.
Abdel- Tawab Khalil: played a vital role through supervising the statistical analysis of the research and reviewing of the manuscript.
Ahmed Hassan: participated by presenting ideas to produce the research in the best way.
Rabie Abdel Aziz: has an effective role in drawing blood samples from cows, reviewing the research, and putting forward good ideas to present the research in the best way.
Ahmed Badr: participated in the statistical analysis of the research results.

Conflict of interest

All authors declare no conflict of interest

Funding

The research received no grants or funds

References

1. Daetwyler, H. D., Capitan, A., Pausch, H., Stothard, P., Van Binsbergen, R., Brøndum, R. F., Liao, X., Djari, A., Rodriguez, S. C., and Grohs, C. (2014). Whole-genome sequencing of 234 bulls facilitates mapping of monogenic and complex traits in cattle. Nature genetics, 46(8): 858-865.
2. Ramos, A. F., and Silva, B. D. M. (2018). Hormonal protocols in small ruminants.
3. González-Bulnes, A., Baird, D. T., Campbell, B. K., Cocero, M. J., García-García, R. M., Inskeep, E. K., López-Sebastián, A., McNeilly, A. S., Santiago-Moreno, J., and Souza, C. J. (2004). Multiple factors affecting the efficiency of multiple ovulation and embryo transfer in sheep and goats. Reproduction, Fertility and Development, 16(4): 421-435.
4. Fukaya, S., Yamazaki, T., Abe, H., Nakagawa, S., Baba, T., Bai, H., Takahashi, M., and Kawahara, M. (2024). Characterization of conception rate after embryo transfer in comparison with that after artificial insemination in dairy cattle. Journal of dairy science, 107(11):9516-9526.
5. Gizaw, Y., Bekana, M., and Abayneh, T. (2007). Major reproductive health problems in smallholder dairy production in and around Nazareth town, Central Ethiopia. Journal of Veterinary Medicine and Animal Health, 5(4):112-115.
6. Portin, P., and Wilkins, A. (2017). The evolving definition of the term "gene". Genetics, 205(4): 1353-1364.
7. Gerstein, M. B., Bruce, C., Rozowsky, J. S., Zheng, D., Du, J., Korbel, J. O., Emanuelsson, O., Zhang, Z. D., Weissman, S., and Snyder, M. (2007). What is a gene, post-ENCODE? History and updated definition. Genome research, 17(6): 669-681.
8. Zhu, J., He, F., Hu, S., and Yu, J. (2008). On the nature of human housekeeping genes. Trends in genetics, 24(10): 481-484.
9. Glare, E. M., Divjak, M., Bailey, M., and Walters, E. H. (2002). -Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax, 57(9):765-770.
10. Dickinson, S. E., Griffin, B. A., Elmore, M. F., Kriese-Anderson, L., Elmore, J. B., Dyce, P. W., Rodning, S. P., and Biase, F. H. (2018). Transcriptome profiles in peripheral white blood cells at the time of artificial insemination discriminate beef heifers with different fertility potential. BMC genomics, 19:1-11.
11. Abdel Aziz, R. L., Hussein, M. M., El Said, H., Kamel, S., Ibrahim, M. A., and Abdel Wahab, A. (2023). Monitoring of health status, performance and transcript abundance of some genes in dairy heifers produced by embryo transfer or artificial insemination. Reproduction in domestic animals, 58(8):1146-1155.
12. Aziz, R. A., Khalil, A., Abdel-Wahab, A., Hassan, N., Abdel-Hamied, E., and Kasimanickam, R. (2017). Relationship among circulating anti-Müllerian hormone, insulin like growth factor 1, cadmium and superovulatory response in dairy cows. Theriogenology, 100: 72-79.
13. Abdel Aziz, R. L., Hussein, M. M., Mohamed, M. A., Elsaid, H., and Abdel Wahab, A. (2022). Heat stress during critical windows of the oestrous cycle and risk of pregnancy establishment in embryo recipient dairy heifers. Reproduction in domestic animals, 57(8): 856-863.
14. IETS, 2010. International embryo transfer society.
15. Järvå, M. A., Lingford, J. P., John, A., Soler, N. M., Scott, N. E., and Goddard-Borger, E. D. (2020). Trefoil factors share a lectin activity that defines their role in mucus. Nature Communications, 11(1):2265.
16. Moorey, S. E., and Biase, F. H. (2020). Beef heifer fertility: importance of management practices and technological advancements. Journal of animal science and biotechnology, 11(1):97.