Braz. J. vet. Res.anim.Sci.,
São Paulo, v. 39, n. 4, p.208-212, 2002.

Relative expression of insulin like growth factor I (IGFI) and follicle stimulating hormone receptor (FSHR) in follicles and ovarian tissue from *Bos primigenius indicus* (Nelore)

CORRESPONDING AUTHOR:
JOSÉ FERNANDO GARCIA
Laboratório de Bioquímica e Biologia Molecular Animal - UNESP
Rua Clóvis Pestana, 793 - Jardim Dona Amélia
16050-680 - Araçatuba – SP
e-mail: jfgarcia@terra.com.br

1- Laboratório de Reprodução Suína e Tecnologia de Sêmen da Universidade Estadual do Ceará, Fortaleza – CE
2- Laboratório de Bioquímica e Biologia Molecular Animal da UNESP, Araçatuba - SP

Expressão relativa de fator semelhante a insulina (IGFI) e receptor do homômonio folículo estimulante (FSHR) em folículos e tecido ovariano de *Bos primigenius* (Nelore)

Jorge Luís FERREIRA[1], Ricardo TONIOLLI[1], Ana Beatriz Graça DUARTE[1], Francine CAMPAGNARI[2], Ariadne Padovez BOSCARO[2], Fabiane Siqueira PAZINI[2], José Fernando GARCIA[2*]

SUMMARY

The improvement of techniques to maximize reproductive potential of females needs the whole comprehension of the controlling mechanisms of follicular development. An alternative for this purpose is the quantification of relative gene expression from those genes involved in recruitment, selection and follicular development, using reverse transcriptase – polymerase chain reaction (RT – PCR). The present study aimed to quantify relative gene expression from insulin-like growth factor I (IGF-I) and follicle stimulating hormone receptor (FSHR) in cattle (*Bos primigenius indicus*), using as internal control the gliceraldheyde 3 phosphate dehydrogenase (GAPDH) gene. Ovaries in different estral cycle stages were obtained from slaughtered animals. Total RNA from follicles and ovarian tissue was purified and the RT-PCR conditions were standardized. PCR products were analyzed in ethydium bromide agarose gels and the bands submitted to densitometric analysis. Exponential amplification curves were constructed and the method's validation was performed using regression analysis to determine the amplification coefficient (E) for each of the three genes. Relative expression for each gene was calculated using the formula described by Prelle *et al.*[12]. In every sample there was gene expression detected for each gene showing differences related to cycle temperature. Amplification coefficient (E) was similar between control gene (GAPDH) and IGFI, independently of the class analyzed. IGFI linear amplification could be related to the constitutive characteristic of this protein since the transcripts are not dependents of FSH levels. It was observed difference in FSHR mRNA expression between the classes analyzed. It could be due to the variation of the receptor number in granulosa cells for each different phase of estral cycle. Semi-quantitative RT-PCR has a large application in biotechnology since it is useful to help us the better understanding of follicular dynamics. However these studies must be conducted using other genes in order to provide new clues for the physiology of folliculogenesis.

KEY-WORDS: Semi-quantitative RT-PCR. Gene expression. Cattle.

INTRODUCTION

Better understanding of the regulating mechanisms between luteal function and follicular development allows more consistent human interference in cattle reproductive function aiming the maximum benefits[9]. Follicular development is constantly regulated by rytmic waves of gonadotrophic hormones and growth factors. Several studies have been conducted aiming to determine the role of these hormones and peptide factors in follicular dynamics [3,14]. Insulin like growth factor I (IGF-I) is a pituitary regulator of follicular growing potentiallizing the action of gonadotrophins in the ovary[1,17]. IGF-I also plays in granulosa cells stimulating their proliferation, promoting steroidogenesis and foliculogenesis, ovulation, fertilization, implantation and subsequent embryo development [11,18].

In situ hybridization has been showing that many of the IGF system proteins (other subtypes, ligands and receptors) are expressed in rat ovaries. Previous reports indicate discrepancy between the site of synthesis and presumable function of these substances in the follicular development. Type I receptor has been demonstrated expressed in granullosa cells either in healthy follicles or atresic, from primary up to pre-ovulatory stages [2]. mRNA expression has been demonstrated as restricted to granullosa cells compartment in healthy follicles in growing phase[16]. However, Zhou et al.[19], report the IGF-I mRNA in granullosa cells as not dependent of FSH levels. Armstrong et al.[3] using in situ hybridization did not detect IGF-I mRNA in granullosa and teca cells in follicles under different stages of development.

The role of FSH in folliculogenesis is well described for all livestock species, however some points of the regulation of its receptor in ovarian cells still remains unknown[10]. Nakamura et al.[12] demonstrated that activin increases the levels of FSHR mRNA in granullosa cells, suggesting that the expression level of this gene increases during follicular development. Xu et al.[15] observed that FSHR mRNA level in ovarian follicles is not related with follicular growth in cattle. In general the FSHR mRNA increasing is followed by concomitant increase of FSH levels, in spite of description of the existence of undefined moments when this relation can be broken[10]. Normally the expression of receptors and steroidogenic enzymes increase during follicular development reaching the maximum rates when it reaches the larger diameter close to the ovulation[7,8]. Recent studies[4,5,6] have been demonstrating that FSHR mRNA is localized in granullosa and cumulus cells. Its expression was localized in primary follicles and, is presumable that it is related with inner teca cells formation and basal membrane enlargement. In addition the intensity of expression for FSHR was similar in pre-antral follicles and in those of small diameter.

Molecular techniques that allow the investigation of differential gene expression could help in the elucidation of the genes involved in folliculogenesis. This study had the objective to verify the mRNA relative expression of IGF-I and FSHR genes in ovarian follicles from slaughtered *Bos primigenius indicus* at different stages using semi-quantitative RT-PCR.

MATERIAL AND METHOD

Ovary sampling

Ovaries from slaughtered *Bos primigenius indicus* females at different stages of estral cycle were recovered. Immediately the follicles were individualized and dissected manually with forceps and scissors in refrigerated benchtop at 4ºC. Prior dissection the follicles were classified as large (8-10 mm), medium (4-7 mm) and small (3 mm). Ovarian tissue fragments containing stroma, follicles and corpora lutea were also collected. All samples were stored at liquid nitrogen.

Total RNA purification

Total RNA from follicles and ovarian tissues was purified using Trizol (Invitrogen), following the manufacturer instructions. The pellet was diluted in 20 mL DEPC treated water and submitted to Dnase I treatment (Amersham Pharmacia Biotech). Total RNA was quantified by spectrophotometric determination and stored at –80ºC.

Reverse transcriptase – polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized using the *Superscript™ First – Strand Synthesis System for RT-PCR* (Invitrogen) that synthesized the first strand of cDNA. All cDNAs were produced using 4 mg of total RNA primed with oligodT, following the manufacturer recommendations.

Gene specific PCR

cDNA samples were submitted to gene specific amplification in a thermocycler (PTC-100 MJ-Research) in order to produce 144, 196 e 850bp DNA fragments from FSHR, IGF-I e GAPDH genes respectively. Primers for FSHR, IGF-I e GAPDH are shown in Table 1. Each cDNA sample was added of the following reagents: dNTPs (1,25 mM each) (Invitrogen), 20 pmol of each primer (forward and reverse) (Invitrogen), 1 UI Amplitaq GoldÒ (Applied Biosystems), Buffer II (10 mM Tris-HCl (pH=8,3), 5 mM KCl) (Applied Biosystems), 2,5 mM $MgCl_2$ (Applied Biosystems) and water (Invitrogen) q.s.p. 25 mL. All samples were heated at 95ºC during 5 min. and PCR amplified for 18, 22, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45 and 48 cycles (each cycle consisted of: 94ºC/45 s - denaturation, 55ºC/45 s – annealing, 72ºC/1.5 min. – extension). At the end of the cycling all samples were submitted to a final extension step of 72ºC/10 min. Exponential amplification curves were generated for each gene.

Agarose gel electrophoresis

Aliquot (7 mL) of each PCR was analyzed in a ethydium bromide stained (5 mg/mL) 1,5 % agarose gel electrophoresis (Invitrogen), during 1 hour at 100 V in Tris-boric acid-EDTA buffer (Invitrogen). Gels were photographed in a UV transiluminator and the bands submitted to densitometric analysis using the software *Kodak Digital Science™ ID Image Analysis.*

mRNA quantification

FSHR, IGF-I and GAPDH were amplified in all samples permitting the construction of exponential amplification curves. Two mathematical methods were employed for the relative mRNA concentration determination: amplification coefficient (E) and relative mRNA expression (ErmRNA). Amplification coefficient (E) was calculated using the formula: $E = 10^{-(1/b)} - 1$, (where b = regression coefficient between band intensity and number of amplification cycles needed for the amplicon concentration curve stabilization). Method's accuracy was determined by the regression coefficient for each tissue class in each analyzed gene.

The second method used for this analysis was the formula proposed by Prelle et al.[13], where the relative mRNA expression was normalized by the concentration of control gene mRNA (GAPDH). Relative mRNA expression (ErmRNA) for each gene was determined using the formula:

$$ErmRNA = \frac{(1 + E_{(I)})^{-CT(I)}}{(1 + E_{(GAPDH)})^{-CT\,(GAPDH)}}$$

where CT = number of amplification cycles needed for the amplicon concentration curve stabilization, I = gene of interest.

Statistical analysis

Data on relative mRNA expression for each gene, obtained using the formula proposed by Prelle et al.[13], was

compared using Duncan's test (P<0.05). Relative mRNA expression data obtained by the difference between the average of number of amplification cycles needed for curve stabilization in each tissue class was tested ANOVA, and the difference either between the genes or the tissue classes was tested by Duncan's test (P<0.05).

RESULTS

FSHR, IGF-I and GAPDH were expressed in all tissue classes analyzed, showing differences in number of amplification cycles needed for the amplicon concentration curve stabilization. Data on amplification coefficient (E) of mRNA for each gene in different tissues are presented in Tab. 2. The results demonstrate no differences between tissue classes for GAPDH and IGF-I expression. However FSHR expression revealed differences among the analyzed tissue classes.

Fig. 1, 2 and 3 illustrate the PCR amplification curves for GAPDH, IGF-I and FSHR respectively. It could be observed variation of the in number of amplification cycles needed for the amplicon concentration curve stabilization in different classes.

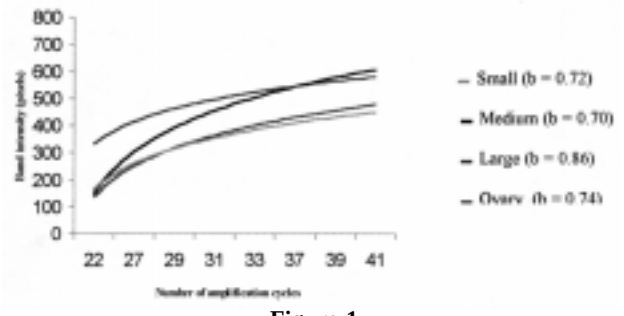

Figure 1

PCR amplification curve for GAPDH in different tissue classes (y axis = band intensity and x axis = number of amplification cycles. b = regression coefficient).

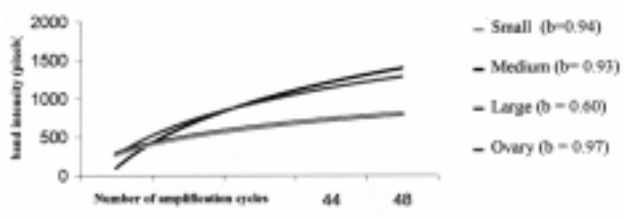

Figure 2

PCR amplification curve for IGF-I in different tissue classes (y axis = band intensity and x axis = number of amplification cycles. b = regression coefficient).

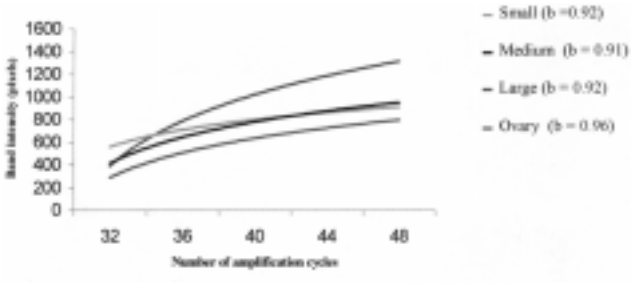

Figure 3

PCR amplification curve for FSHR in different tissue classes (y axis = band intensity and x axis = number of amplification cycles. b = regression coefficient)

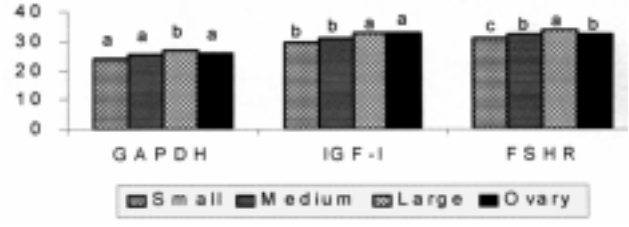

Figure 4

CT value for GAPDH, IGF-I and FSHR in different tissue classes. [a,b,c] different letters between tissue classes in the same gene differs significantly P<0.05.

Table 1

Primers sequences used in the RT-PCR for the genes FSHR, IGF-I e GAPDH.

Gene	Amplicon size (bp)	Primer sequences
FSHR	144	5'ATGGCTGAGTAAGAATGGGATTC3'
		5'AGAATGACCGGTCCAGAGGCTCC3'
IGF-I	196	5'CCTCTGCGGGGCTGAGTTGGT3'
		5'CGACTTGGCGGGCTTGAGAGGC 3'
GAPDH	850	5'TCCACCACCCTGTTGCTG3'
		5'TGTTCCAGTATGATGATTCCACC 3'

Table 2

Amplification coefficient (E) for different tissue classes.

	Tissue Classes			
	Small Follicle	**Medium Follicle**	**Large Follicle**	**Ovarian Tissue**
FSHR	0,91	0,91	0,98	0,90
IGF-I	0,92	0,92	0,92	0,90
GAPDH	0,96	0,93	0,93	0,96

ANOVA test revealed that GAPDH had different expression levels compared with IGF-I and FSHR based on CT in tissue classes analyzed. Although no significant variation between CT average in IGF-I and FSHR, it was observed when comparing these averages by Duncan's test, differences in mRNA expression levels for FSHR on the tissue classes analyzed. The observation of relative mRNA expression for all genes revealed that the small follicles had expression pattern differing to the other tissue classes (Fig. 4).

Relative mRNA expression of FSHR and IGF-I in different tissue classes when analyzed by the method proposed by Prelle et al.[13], showed no interaction between gene and tissue class analyzed.

DISCUSSION

Amplification coefficient (E) for GAPDH and IGF-I in all tissue classes analyzed had similar values, indicating the constant mRNA expression level for both genes. Similar results were found when relative expression was determined by the method proposed by Prelle et al.[13]. However, CT analysis for IGF-I and GAPDH showed discrepancy in mRNA expression between them in the different tissue classes, indicating the constitutive character of GAPDH in different physiological situations. Results observed for IGF-I lead us to conclude that the this protein is constitutively produce be granullosa cells, showing similarity in expression between different follicle classes. Zhou et al.[19] that imply mRNA expression of IGF-I in granullosa cells not dependent of FSH levels corroborates these data. However these data are different from those described by Armstrong et al. [3] that did not show IGF-I mRNA in bovine follicles.

FSHR mRNA expression data collected in this study revealed either amplification coefficient (E) or EmRNA value differing significantly between the tissue classes analyzed ($P<0.05$). These results agree with the general idea that expression level of transmembrane receptors and steroidogenic enzymes increases during follicular development, reaching the maximum stage when the follicle has the larger diameter, close to the ovulation[7,8]. Also the results for FSHR could be explained because the increasing of FSHR mRNA is followed by concomitant increase of FSH levels[10]. Other possible explication could be the variation on the number of receptors in granullosa cells on different cell types as described previously[12].

These results indicated the importance of semi-quantitative RT-PCR to help understand physiology processes, mainly in the dynamic system of ovarian follicle growth. However these studies should be amplified in order to analyze other genes and in other tissue classes to help the understanding of ovulation processes in cattle.

ACKNOWLEDGEMENTS

Financial Support: Fundação de Apoio à Pesquisa do Estado de São Paulo –FAPESP. Technical assistance and fruitful discussions: J. Buratini Júnior and A. Teixeira – UNESP– Department of Physiology – Botucatu – Brasil, F. V. Meirelles – Universidade de São Paulo – Faculdade de Zootecnia e Engenharia de Alimentos – Pirassununga – Brasil, T. C. Cardoso da Silva and N. Fachin – UNESP – Araçatuba.

RESUMO

O aperfeiçoamento das técnicas que objetivam a exploração do potencial reprodutivo das fêmeas requer a compreensão mais ampla dos mecanismos de controle de desenvolvimento folicular. Uma alternativa de estudo nesta esfera, é a quantificação da expressão relativa de genes envolvidos nos processos de recrutamento, seleção e desenvolvimento folicular, pelo emprego da técnica de transcrição – reversa associado a reação em cadeia pela polimerase (RT – PCR). O presente trabalho objetivou quantificar a expressão relativa dos genes insulin-like growth factor I (IGF-I) e do receptor do hormônio folículo estimulante (FSHR), tendo como controle interno o gene da gliceraldeído 3-fosfato desidrogenase (GAPDH). Foram utilizados ovários bovinos de animais de matadouro em diferentes fases do ciclo estral. O RNA total dos folículos e tecido ovarianos foi purificado por TRIZOL. As reações de RT-PCR foram realizadas com o "kit" *SuperScript™ First-Strand*. Os produtos de PCR foram analisados em gel de agarose e as bandas submetidas à análise densitométrica. Todos os genes foram amplificados observando-se a curva exponencial de amplificação, a validação do método foi realizada através de análise de regressão, sendo estabelecido o coeficiente de amplificação (E). A expressão relativa de mRNA para cada gene de interesse foi calculada pela fórmula estabelecida por Prelle *et al.*[12]. Em todos os tecidos analisados, todos os genes foram expressos, sobressaltando-se diferenças nos diferentes ciclos estudados. Com relação os dados referentes ao coeficiente de amplificação (E), observou-se tanto para gene controle (GAPDH), como para o gene IGF-I concordância nos valores encontrados para as diferentes classes analisadas. Quanto ao gene IGF-I, a interpretação dos achados para a expressão relativa de mRNA pode está relacionada ao caráter constitutivo dessa proteína ou devido os transcritos não serem dependentes dos níveis de FSH. Observou-se diferenças na expressão relativa de mRNA de FSHR entre as classes de tecidos analisados, o que pode ser explicado pela variação do número de receptores nas células da granulosa nas diferentes fases do ciclo estral. Pode-se perceber a partir desse estudo que a técnica de RT-PCR semi quantitativo é de grande importância biotecnológica, possibilitando auxílio na compreensão da dinâmica folicular. Entretanto esses estudos devem ser ampliados com outros genes para melhor compreensão das etapas fisiológicas envolvidas na foliculogênese.

PALAVRAS-CHAVE: RT-PCR semi-quantitativo, expressão gênica, bovinos.

FERREIRA, J.L.; TONIOLLI, R.; DUARTE, A.B.G.; CAMPAGNARI, F.; BOSCARO, A.P.; PAZINI, F.S.; GARCIA, J.F. Relative expression of insulin like growth factor I (IGFI) and follicle stimulating hormone receptor (FSHR) in follicles and ovarian tissue from *Bos primigenius indicus* (Nelore). **Braz. J. vet. Res. anim. Sci.**, São Paulo, v.39, n.4, p. 208-212, 2002.

REFERENCES

1. Adashi, E. Y.; Roban, M. Intraovarion regulation. Peptidergic signalling systems**. Trends in Endocrinology and Metabolism**, (3):243.248, 1992.

2. Adashi, E. Y.; Resnick, C. E.; Payne, D. W.; Rosenfeld, R. G.; Matsumoto, T.; Hunter, M. K.; Gargosky, S. E.; Zhou, J.; Bondy, C.A. The mouse intraovarian insulin-like growth factor I system: departures from the rat paradigm. **Endocrinology**, (138):3881-3890, 1997.

3. Armstrong, D. G.; Gutierrez, C. G.; Baxter, G.; Glazyrin, A. L.; Mann, G. E.; Woad, K. J.; Hogg, C. O.; Webb, R. Expression of mRNA encoding IGF-I, IGF-II and 1 IGF receptor in bovine ovarian follicles. **J. Endocrinol**. (165):101-113, 2000.

4. Bao, B., Calder, M. D., Xie, S., Smith, M. F., Youngquist, R. S., Garverick, H. Steroidogenic acute regulatory protein messenger ribonucleic acid (mRNA) expression is limited to theca of healthy bovine follicles. **Biol. Reprod**. 56 (Supl.10), 143, 1997.

5. Bao, B., Garverick, H. A., Smith, M. F., Smith, B. E. S., Youngquist, R. S. Changes in messenger ribonucleic acid (mRNA) encoding LH receptor, cytochrome P450 side chain cleavage and aromatase are associated with recruitment and selection of bovine ovarian follicles. **Biol. Reprod**., 56, 1158-1168, 1997.

6. Bao, B., Garverick, H. A., Smith, M. F., Smith, B. E. S., Youngquist, R. S. Expression of messenger ribonucleic acid (mRNA) encoding 3ß-hydrogenase dehydroxysteroid during recruitment and selection of bovine ovarian follicles. **Biol. Reprod**., 56, 1466-1473, 1997.

7. Bao, B.; Garverick, H. A. Expression of steroidogenic enzyme and gonadotrophin receptors genes in bovine follicles during ovarian follicular waves: A review. **J.Anim.Sci.**,76, 1903-21, 1998.

8. Evans, A. C. O.; Fortune, J. E. Selection of the dominant follicle in cattle occurs in the messenger ribonucleic acid or gonadotrophin receptors. **Endocrinology**, 138, 2963-71, 1997.

9. Figueiredo, R. A.; Barros, C. M.; Rocha, G. P.; Papa, F. O. Prevalência de duas ondas de crescimento folicular ovariano em vacas da raça Nelore. **Rev. Bras. Reprod. Anim**. 19(3-4), 200-211, 1995.

10. Findlay, B. R.; Drummond, A. E. Regulation of the FSH receptor in the ovary. **Trends Endocrinol. Metabolism**, 10(5), 183-188, 1995.

11. Lackey, B. R.; Gray, S. L. L.; Henricks, D. M. Physiological basis for use of insulin-like growth factors in reproductive applications: a review. **Theriogenology**, (53), 1147-1156, 2000.

12. Nakamura, M.; Minegishi, T.; Hasegawa, Y.; Nakamura, K.; Igarashi, S.; Ito, I.; Shinozaki, H.; Miyamoto, K.; Eto, Y.; Ibuki, Y. Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. **Endocrinology** 133, 538-544, 1993.

13. Prelle, K.; Stojkovic, M.; Boxhammer, K.; Motlik, J.; Ewald, D.; Arnold, G. J.; Wolf, E. Insulin-like growth factor I (IGF-I) and long R^3IGF-I differently affect development and messenger type I IGF receptors in vitro produced bovine embryos. **Endocrinology** 142(3), 1309-1316, 2001.

14. Soto, R. L.; Simmem, F. A.; Diaz, T.; Thatcher, W. W. Insulin-like growth factor system in bovine first-wave dominant and subordinate follicles. **Biol. Reprod**. (53), 1359-1364, 1996.

15. Xu, Z. Z.; Garverick, H. A.; Smith, M. F.; Smith, S. A. H.; Yongquist, R. S. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. **Biol. Reprod.** (53):951-957, 1995.

16. Wandji, A. S.; Wood, T. L.; Crawford, J.; Levison, S. W.; Hammond, J. M. Expression of mouse ovarian insulin growth factor system components during follicular development and atresia. **Endocrinology** (139):5205-5214, 1998.

17. Webb, R.; Armstrong, D. G. Control of ovarian function effect of local interactions and enviromental influences on follicular turnover in cattle: a review. **Livestock Production Science** 53, 95-112, 1998.

18. Yoshimura, Y. Insulin-like growth factors and ovarian physiology. **J. Obstet. Gynaecol. Res.** 24, 305-323, 1998.

19. Zhou, J.; Kumar, T. R.; Matzuk, M. M.; Bondy, C. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. **Mol. Endocrinol**., 11:1924-1933, 1997.

Received: 04/12/2001
Accepted: 21/05/2002