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The effects of follicle diameter on the *in vitro* fertilization capacity of bovine oocytes aspirated from the slaugthered ovaries

Mustafa ÜN, Şükrü KÜPLÜLÜ

Department of Gynaecology and Obstetrics, Faculty of Veterinary Medicine, Ankara University, Ankara

Summary: The aim of this study was to determine the maturation and fertilization capacities of bovine oocytes aspirated from slaugthered ovaries in relation with the follicle diameter. A number of 115 ovaries collected from the slaugthered cows at a local slaughterhouse were used as the material. Peripheral follicles were counted according to the their diameters (2-6 mm-group I and 6-10 mm-group II). All follicles were punctured with an 18 G needle hold on a 5 ml syringe and aspirated cumulus-oocyte complexes were classified in regard to their morphological apperance. Only Grade I, II and III oocytes were then placed in maturation medium (TCM-199+%20 (v/v) ECS+BSA) and incubated under an atmosphere of 5% CO_2 at 39°C for 22-24 hr. After IVM, maturated oocytes were fertilized by adding 1-2 µl ($1x10^6$), swim-up separated sperm to the fertilization media (Tyrode's albumin lactate pyruvate medium-TALP) for *in vitro* fertilization under an atmosphere of 5% CO_2 at 39°C for 18-19 hr. Heparine (10 µg/ml) was used as the capacitating agent. The datas obtained at the all stages were recorded and statistical evaluation was done with the Student's T test. A total of 549 (4.77±2.09) oocytes were aspirated from 588 (5.11±2.39) follicles with an aspiration rate of 93.3% in group I and 275 (2.5±1.87) COC were aspirated from 300 (2.72±1.58) follicles with an aspiration rate of 91.7% in group II. After the maturation period 401 of 549 oocytes in group I and 217 of 275 oocytes in group II were found as mature with a maturation rate of 73% and 78.9%, respectively ($p>0.05$). In 165 of 401 in group I (41.1%) and 107 of 217 incubated oocytes in group II (49.3%), both male and female pronuclei were detected. As a conclusion, it was evident that the ovaries collected from the slaugtherhouse are sufficient potentials for *in vitro* embryo production, although a great variation between the maturation and fertilization capacities of oocytes aspirated from the peripheral follicles could be observed. It was also obvious that there is a significiant relation between the follicle diameter and maturation and fertilization capacities of oocytes since the fertilization rates increases as the follicle diameter rises.

Key words: Bovine, fertilization, *in vitro*, maturation, oocyte

Mezbahadan toplanan ovaryumlardan aspire edilen sığır oositlerinin *in vitro* fertilizasyonu üzerine follikül büyüklüklerinin etkisi

Özet: Bu çalışmada, mezbahadan toplanan ovaryumlardan elde edilen sığır oositlerinin maturasyon ve fertilizasyon oranlarının follikül çapı ile ilişkilendirilerek ortaya konması amaçlanmıştır. Çalışma materyali olarak bölge mezbahalarında kesilen hayvanlardan toplanan 115 ovaryum kullanıldı. Ovaryumların üzerlerindeki yüzeysel folliküller çaplarına göre (2-6 mm-Grup I ve 6-10 mm-Grup II) sayıldı. Tüm folliküllerin 18 G'lik iğne ile punksiyonları yapılarak oositler aspire edildi. Aspire edilen kumulus-oosit kompleksleri morfolojilerine göre sınıflandırıldı. Yanlızca I. ve II. kalite oositler *in vitro* maturasyon vasatına (TCM-199+%20 (v/v) ECS+BSA) aktarılarak, 39°C sıcaklıkta %5 CO_2 atmosferinde 22-24 saat inkübe edildi. İnkubasyon sonrası perivitellin boşlukta Iç kutup hücresi ve kumulus ekspansiyonu görülen oositler mature olarak kabul edilerek *in vitro* fertilizasyon vasatına (Tyrode'nin albumin laktat piruvat vasatı-TALP) aktarıldı. Fertilizasyon swim-up testi ile immotil spermatozoon populasyonundan ayrıştırılmış, final konsantrasyonu 50x106 spermatozoon/ml olan spermadan 1-2 µl'nin ($1x10^6$) fertilizasyon vasatına aktrarılması ve vasata heparin (10 µg/ml) eklenerek kapasitasyonun sağlanması ile 39°C sıcaklıkta %5 CO_2 atmosferinde 18-19 saatte gerçekleştirildi. Çalışmanın her basamağına ait veriler kaydedilerek Student's K testi ile istatistiki değerlendirmesi yapıldı. Çalışma sonucunda Grup I'de yer alan 588 (5.11±2.39) ve Grup II'de yeralan 300 (2.72±1.58) follikülden aspire edilen toplam ve ovaryum başına ortalama oosit sayıları sırasıyla 549 (4.77±2.09) ve 275 (2.5 ± 1.87) olarak kaydedildi. Aspirasyon başarıları ise Grup I'de %93.3 ve Grup II'de %91.7 olarak hesaplandı. Grup I'de aspirasyondan sonra I ve II. kalite oldukları belirlenen toplam 549 ve Grup II'de 275 cumulus-oosit kompleksinin maturasyon kültürü sonrası Grup I'de 401 ve Grup II'de ise 217'sinde maturasyonun şekillendiği saptandı. Grup I ve II'de elde edilen maturasyon yüzdeleri ise sırasıyla %73 ve %78.9 olarak belirlendi ($p>0.05$). Grup I ve II'ye ait mature oositler *in vitro* fertilizasyon işlemi için kullanıldı. Bu oositlerden Grup I'de 165 (%41.1)'inde ve Grup II'de 107 (%49.3)'sinde, fertilizasyon kültürü sonrası mikroskobik incelemede, hem erkek hem de dişi pronukleus görülerek fertilize oldukları kabul edildi Sonuç olarak, mezbahadan toplanan sığır ovaryumları *in vitro* embriyo üretiminde iyi bir kaynak olduğu, ancak toplanan ovaryumların yüzeyindeki folliküllerden aspire edilen oositlerin maturasyon ve fertilizasyon kapasiteleri oldukça değişkenlik gösterdiği ve *in vitro* çalışmalarda kullanılabilecek oositlerin elde edildikleri folliküllerin çapları ile oositlerin maturasyon ve fertilizasyon başarıları arasında kuvvetli bir ilişki olduğu, follikül ölçüsü arttıkça fertilizasyon oranının yükseldiği sonucuna varıldı.

Anahtar kelimeler: Fertilizasyon, *in vitro*, maturasyon, oosit, sığır

Introduction

In vitro bovine embryo production has improved tremendously during the past decade and numerous research and practical applications have been developed for the production of bovine embryos *in vitro* (1,2). The primary techniques essential for embryo production are maturation of oocytes *in vitro* (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culture (3,4,9). Combining these techniques enables the large scale production of mature and fertilized oocytes for the introduction of genes, embryos for the production of embryonic stem cells and embryos after transfer, and oocytes and embryos for embryo transfer (12,14). The available statistics published by International Embryo Transfer Society show that the use of embryo transfer technology has increased rapidly during 1980's and the early 1990's (6). However, embryo production has stabilized over the past 5 years. Part of the reason for this plateau in the use of embryo transfer is the difficulty to harmonize this technology with the production goals of every herd (14).

The efficiency of embryo production to morula or blastocyst stages is still low (10,11). Although *in vivo* conditions may never be duplicated *in vitro*, oocyte maturation and also fertilization can be improved by experimentation with medium and supplements- especially the sera (18). The maturation of the oocyte is a complex phenomenon involving both the nucleus and the cytoplasm. Thompson (17) has stated the fact that there is a positive relation between the follicle diameter and the maturational capacity. Similarly, Gordon (6) explained this manner with the oocytes grow-up. Contrary, some authors (16,18,19) stated a disagreement to the sited phenomen.

The objective of this study was to demonstrate that IVF technology can be an effective solution for upgrading the cattle population and to determine the effectiveness of the mostly used IVF techniques by the means of oocyte maturation and fertilization in relation with the follicle diameter.

Materials and Methods

Ovary collection

A total of 482 ovaries were collected from the local slaughterhouse immedietaly after the culling of the cow and placed in a 500 ml-thermos filled with saline including peniciline (100 IU/ml) and amphotericine-B (50 µg/ml). Transportation duration was aimed to be minimized and ranged between 2-4 h.

Oocyte aspiration

Cumulus-oocyte complexes (COC) were obtained by aspiration of 2 to 7 mm (classified with compass) follicles (group I) and 7-10 mm follicles (group II) with a 10 cc syringe fitted with an 18 gauge neddle and the aspirated follicular fluid was pooled in a 50-ml flask. Cumulus-oocyte complexes having a sandy cytoplasm and at least 2-4 layers of cumulus cells were chosen for *in vitro* maturation.

In vitro maturation

Selected oocytes were placed in culture medium (TCM-199) (cat. no.M 2520, Sigma GmbH, Europe) added with ECS (20% v/v) and 0.6% BSA with a population of 10 oocytes in 50 µl microdroplets, covered with aliquot volume of sterile mineral oil. Cultures in study groups were done at 39°C and 5% CO_2 in humidified air for 24 h. After the incubation period, oocytes showing the first polar body in perivitelline space and expanded cumulus cells were recorded and considered as matured.

In vitro fertilization

Before fertilization, cumulus cells were removed by pipetting with a large bore pipette (350 µm i.d.). Fertilization medium was TALP modified by reducing Ca^{++} (Ca-free TALP). Oocytes were fertilized using methods similar to those described by Gordon (6). Briefly, sperm that had been frozen and then thawed were layered under 2 ml of sperm-TALP and incubated with an 45°-standing position for 1 hr at 39°C and 5% CO_2 in air. The upper layer of the tube was then collected (0.8 ml) and suspended with equal volume of sperm-TALP to a final concentration of $50x10^6$ motile sperm/ml. Fertilization was achieved by adding 1-2 µl of sperm suspention directly to the fertilization medium (fert-TALP) with the capacitating effect of heparine (10 µg/ml). At 24th hr postinsemination, cleavage rate was determined according to the observation of both two pronuclei.

Statistical analyses

Statistical analyses of all datas obtained from study groups were done with the Student's T-test in SPSS for Windows® programme and significiant differences were managed as 2 digits after comma.

Results

Every 2-7 mm (group I) and 7-10 mm (group II) follicles on the same ovaries (A total of 115 were collected) were used to have the maximum uniformity. The time sequence between the first and the last ovary put into the thermos was 35-180 min (mean 80 min) according to the culling que and density. The alteration of the temperature of transport medium were recorded between 25-35°C and the transport time (i.e. from slaughterhouse to laboratory) was 135-225 min.

Oocyte aspiration

A total of 549 (4.77±2.09) oocytes were aspirated from 588 (5.11±2.39) follicles with an aspiration rate of

93.3% in group I and 275 (2.5±1.87) COC were aspirated from 300 (2.72±1.58) follicles with an aspiration rate of 91.7% in group II (Figure 1, 2 and 3). The follicle numbers and population of oocytes aspirated were found greatest in group I ($p<0.01$). However, no significiant difference were detected in aspiration rates (Table 1).

Table 1. Overall and mean follicle numbers and total and mean numbers of aspirated oocytes with aspiration rates (%).

Parameter	Group I (2-7 mm)	Group II (7-10 mm)
Follicle (n)	588[a]	300[b]
Follicle (mean)	5.11 ± 2.39[c]	2.72 ± 1.58[d]
Aspirated oocyte (n)	549[e]	275[f]
Oocyte (mean)	4.77±2.09[g]	2.5±1.87[h]
Aspiration rate (%)	93.3[i]	91.7[i]

Means within a column followed by different superscript letters differ (a,b,c,d $p<0.01$ and e,f,g,h,ı $p<0.05$).

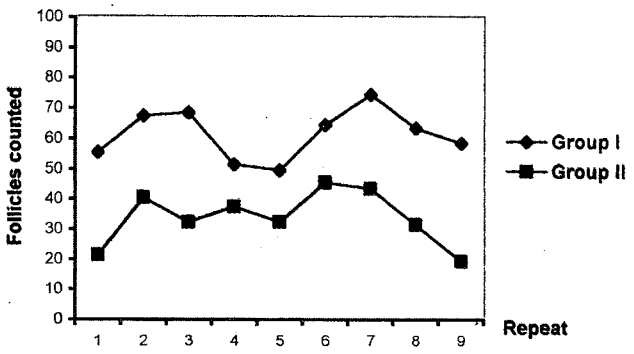


Figure 1. Distribution of follicle counts into repeats in group I and II.

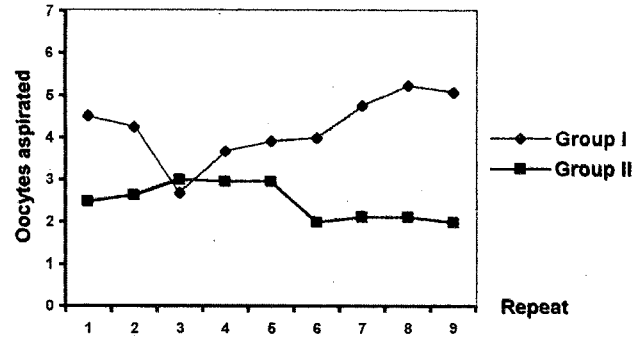


Figure 2. Distribution of mean oocyte counts into repeats in group I and II.

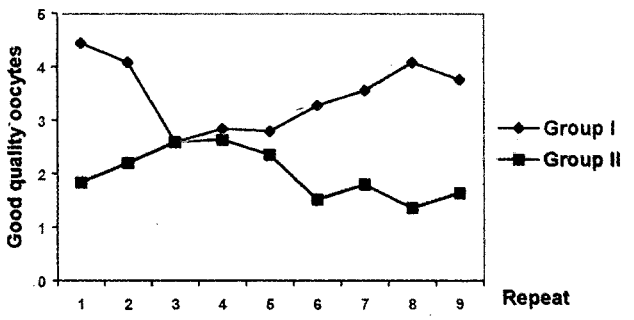


Figure 3. Distribution of good quality COC's into repeats ($p<0.01$).

In vitro maturation

After the maturation period, 401 of 549 selected (good quality) oocytes in group I and 217 of 275 cultured oocytes in group II were matured with a maturation of 73% and 78.9%, respectively (Table 2, Figure 4 and 5), however, aberrant maturation findings were observed in 8% of oocytes in group I and 5.1% of oocytes in group II (Figure 6).

Table 2. *In vitro* maturation results recorded in study groups.

Parameter	Group I (2-7 mm)	Group II (7-10 mm)
Cultured oocyte (n)	549[a]	275[b]
Matured oocyte(n)	401	217
Maturation rate (%)	73%	78.9%
Aberrant maturated oocyte (n)	44±1.3[g]	14±1.17[h]
Aberrant maturation (%)	8%i	5.1%[j]

Means within a column followed by different superscript letters differ (a,b,c,d $p<0.01$ and e,f,g,h,ı,j $p<0.05$).

A significiant difference could not be detected between study groups by the means of maturational capacity ($p>0.05$), but maturation rates differ between the

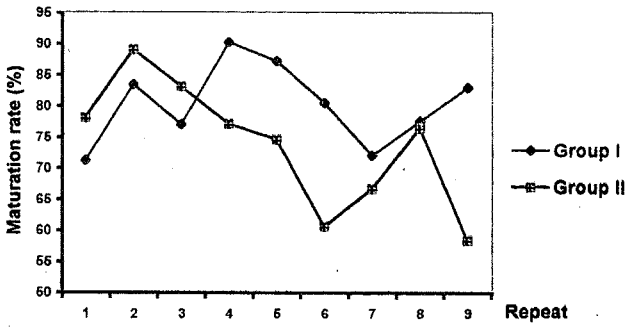


Figure 4. Maturation rates observed in repeats (%).

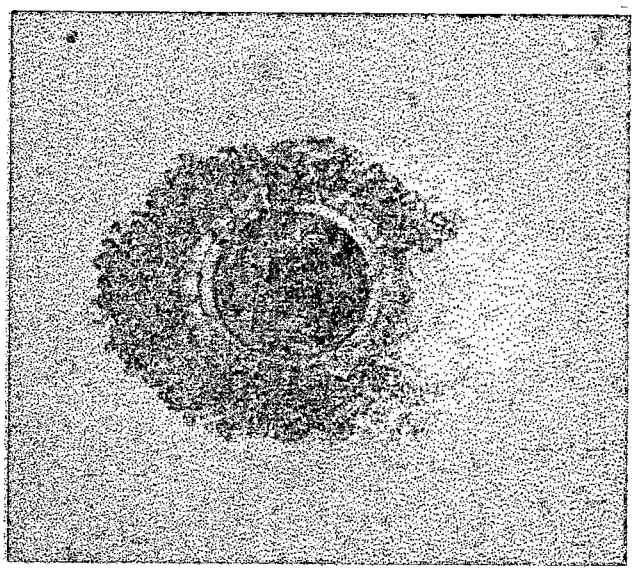
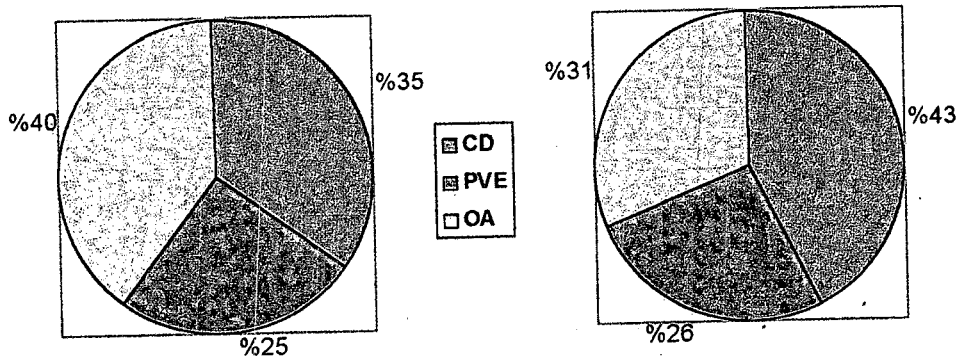


Figure 5. Maturated oocyte.



CD: Cumulus degeneration, PVE: Perivitelline space expantion, OA: Oolemma

Figure 6. Distribution of morphological apperances observed in aberrant maturation.

repeats in the same study group ($p<0.05$) (Figure 9). In addition, aberrant maturation rates were greater in group I compared with group II ($p<0.05$).

In vitro fertilization

In vitro fertilization of 401 (group I) and 217 matured oocytes (group II) has resulted 165 fertilized in group I (41.1%) and 107 fertilized oocytes in group II (49.3%). In group I, 11 were (2.7%) parthenogenetic, polyspermia in 13 oocytes (3.2%) and 3 (0.7%) oocytes showed chromatine degeneration. Same parameters were found as, 6 (2.7%), 10 (4.6%) and 2 (0.9%) in group II (Table 3, Figure 7 and 8).

Table 3. Fertilization results in study groups.

Parameter	Group I (2-7 mm)	Group II (7-10 mm)
Cultured oocyte (n)	401[a]	217[b]
Fertilized oocyte (n)	165	107
Fertilization rate (%)	41.1%	49.3%
Parthenogenesis (n) (%)	11(2.7[i])	6(2.7[j])
Polyspermia (n) (%)	13(3.2)	10 (4.6)
Chromatine degeneration	3 (0.7)	2(0.9)

Means within a column followed by different superscript letters differ (a,b,c,d $p<0.05$ and e,f,g,h,ı,j $p<0.05$).

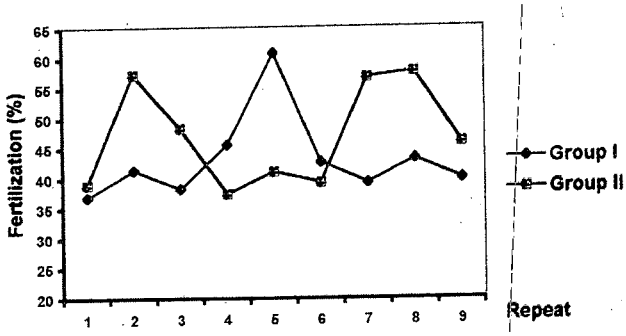


Figure 7. Fertilization rates in study groups and repeats

Fertilization rates were significantly superior in group II to fertilization rates observed in group I ($p<0.05$), however fertilization rates were differ statistically within the repeats of the same groups ($p<0.05$).

Discussion and Conclusion

The importance of ovary collection techniques and transfer expressions were discussed by different authors (6,7,10,11). Leibfried-Rutledge (12) bundled the efficiency of IVF system directly to oocyte recovery conditions-mainly transfer temperature and speed. Gordon (6) stated a

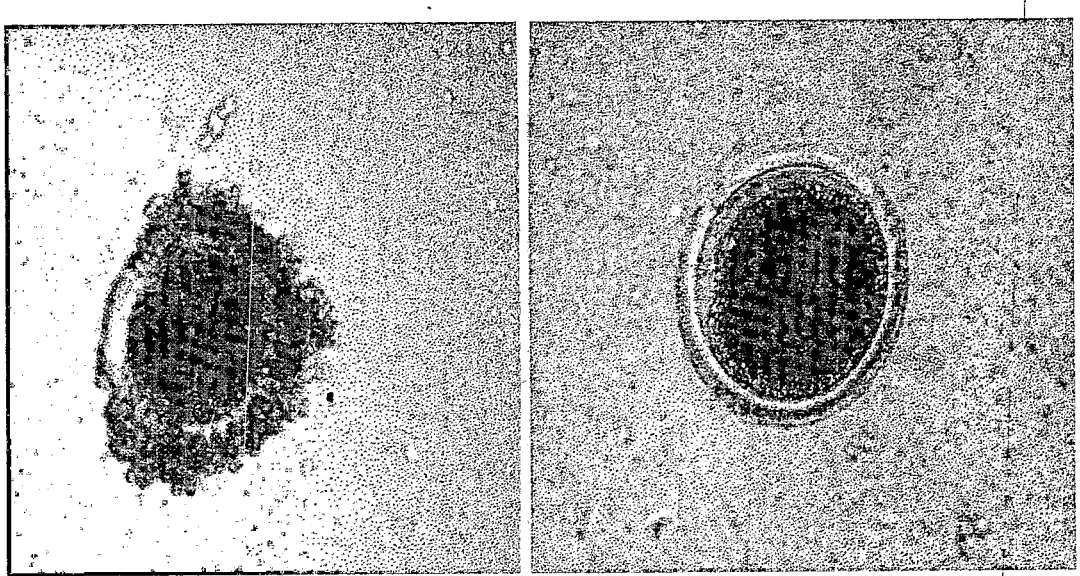


Figure 8. *In vitro* fertilized (pronuclear) oocytes.

positive correlation between ovary transport time and temperature and maturation capacity (60% at <15°C, 85% at >25°C). In the present study, the transport interval and temperature were carefully stabled (app 3 hour and 25-35°C). In order to achieve the satisfactory results suggested by workers above.

Follicle diameter which the oocytes are aspirated from is an important varient and a good sign of the further maturation and fertilization capacities (6). Greve et al. (7) have suggested maturation rates of 85% and 95% for oocytes of follicles <6 and >7 in diameter. Same suggestions were done by Küplülü and Ün (11) (70%-80%) and Telfer (16). All authors discussed this relation with the hormone receptors gained during the oogenesis. Similarly the fact that the lack of oocytes of smaller (<2 mm) follicles achieve the second metaphase was highlighted by Gordon (6). The study of Gordon (6) has shown that there were no presence of LH receptors in such oocytes and the critical point of oogenesis for LH receptors is the reaching a diameter of 3-4 mm. Nevertheless, IVM of bovine oocytes depends upon the synthesis of several distinct and still almost defined proteins culminates the maturational capacities (14). This protein synthesis sequence alters during the follicle development. Gordon (6) suggested a higher protein synthesis in oocytes aspirated from larger follicles (>7 mm) where low at small follicles (3-6 mm).

In the present study, in contrast with the studies above the maturational capacities of both oocytes from small (2-6 mm) and larger (6-10 mm) follicles were not differed (73%-78.9%) significantly. We considered the depud with the maturation tecnique as Jegwnow et al. (8) stated the every similarity of tecniques when making comparision. However the main demonstration of the maturation is fertilization (6,7). Since the fertilization rates of oocytes of large follicles were significantly higher than of smaller ones (49.3% vs 41.1%). Although it was evident that further studies should be done to evaluate the ultrastructural basis of this high differantiation.

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Corresponding address:
Prof. Dr. Şükrü Küplülü
Ankara Üniversitesi Veteriner Fakültesi
Doğum ve Jinekoloji Anabilim Dalı,
Dışkapı, 06110 Ankara.