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Chromosome instability in Mediterranean Italian buffaloes affected by limb malformation (transversal hemimelia)

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For several years, a genetic disease called transversal hemimelia (TH), also known as congenital amputation, has been spreading in Mediterranean Italian buffalo. TH is characterized by the lack of limb distal structures, normally developing proximally to the malformed limb and being amputated at different points distally. A sample of 13 animals affected by TH was examined using the chromosome aberration (CA) test to better characterize chromosome instability already emerging in a preliminary study where we found a significantly higher difference ($P < 0.001$) in the mean rate of sister chromatid exchange/cell (8.80 ± 3.19) performed in 10 malformed animals, when compared with the control (6.61 ± 2.73). The percentage of aneuploid cells was higher in animals with TH (12.76) than in control animals (7.85). Mean gaps are greater in cells of animals with TH (6.62 ± 2.38) than those found in the control (2.86 ± 1.01) and similar results were obtained in chromatid breaks (0.13 ± 0.31 and 0.07 ± 0.06, respectively), chromosome breaks (0.11 ± 0.27 and 0.06 ± 0.13, respectively) and CAs excluding gaps (0.24 ± 0.47 and 0.13 ± 0.18, respectively). All these differences are statistically highly significant ($P < 0.001$).

Introduction

In Italy, buffalo farming has achieved such high production standards that it has become an international point of reference, using innovation breeding technologies, above all in feeding and reproduction. On the back of the business success of buffalo milk products and recently also beef and its products, the increase in the buffalo population is growing despite recurrent health (brucellosis) and environmental (dioxins) crises. In 1999 in Italy, there were ∼165 000 head, in 2008 ∼400 000 head and in 2013, according to Associazione Nazionale Allevatori specie Bufalina—Buffalo Breeders' Association forecasts, there will be 500 000.

The need to increase production due to growing market demand is an incentive both to improve breeding and feeding techniques and for genetic selection. However, pursuing the sole aim of increasing milk production has increased inbreeding in Mediterranean Italian buffalo, which is often the cause of unwanted morphological traits due to genes with negative phenotypic effects.

For several years in the Mediterranean Italian buffalo, a genetic disease has been spreading called transversal hemimelia (TH), a developmental abnormality characterized by the lack of limb distal structures. This malformation is also known as congenital amputation since proximally the malformed limb is normally developed while distally it is amputated at different points (1).

Recently, extensive research has been carried out to discover the molecular mechanisms responsible for embryonic limb development along three main axes. Each axis has a key organizing centre working with the production of specific regulating signals, namely, Sonic hedgehog (Shh) for the anteroposterior; fibroblast growth factors (Fgfs) for the proximodistal and bone morphogenetic proteins (BMPs), engrailed (EN1) and wingless-type MMTV integration site family, member 7A (WNT7A) for the dorsoventral (2).

Abnormalities in the expression of such signals are responsible for embryo limb developmental malformations. Especially, in the human mutation of WNT7A, they have been related to malformations like those reported in buffalo calves with TH (3), while according to other studies carried out on mouse embryos the absence of *Shh* gene activity during embryo hind limb development causes severe malformations in the distal structures from tibia and ulna with the lack of a metatarsus and phalanxes replaced by a single bone (4). Different types of limb malformations have been reported in livestock like paraxial radial hemimelia in goats (5), amelia of thoracic limbs in a calf (6) and tibial hemimelia associated with meningocele and abdominal hernia in shorthorn cattle (7).

To investigate genome stability, the tests mainly used are the evaluation of chromosome aberrations (CAs) and sister chromatid exchanges (SCEs). Both structural and numerical aberrations have been associated with congenital abnormalities in newborn and cancer in humans. Several human recessive diseases such as ataxia telangiectasia, Fanconi's anaemia and Bloom's syndrome are associated with increased chromosomal instability (8). River buffalo (9) and the main livestock species (10–14) have been extensively studied to discover DNA damage caused by a variety of natural and artificial chemical compounds (15–19) and to evaluate chromosome instability associated to congenital malformations (20,21).

In the present study, a sample of 13 animals affected by TH was examined using the CA test to better characterize the chromosome instability already shown in a preliminary study where we found an increased level of SCE in 10 malformed animals.

Materials and methods

The study was carried out on 26 Mediterranean Italian buffaloes (13 males and 13 females) from a few days to 6 months of age, 13 of which were affected by TH (group 1; 6 males and 7 females) and 13 were healthy (group 2; 7 males and 6 females). The buffaloes with TH came from four farms in the southern Italian

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provinces of Salerno, Campobasso, Caserta and Catanzaro, while the healthy animals came from three farms in the provinces of Salerno and Caserta.

Malformed buffaloes were examined and after slaughter necroscopic and radiological (Figure 1) evaluations were performed. Peripheral blood (1 ml) was cultured at 37.5°C in RPMI medium, enriched with foetal calf serum (10%), L-glutamine (1%) and concanavalin A (1.5%) for ~72 h. Cells were harvested after colcemid (0.3 μg/ml) treatment for 1 h and given hypotonic treatment (KCl 0.5%) and three fixations in methanol-acetic acid (3:1), the third overnight. Three drops of cell suspension were air dried on cleaned and wet slides which were stained a day later with acridine orange (0.1% in a phosphate buffer, pH 7.0) for 3 min, washed in tap and distilled water and mounted in the same phosphate buffer. Slides were observed ~24 h after staining or later (1 week). At least 100 cells per animal were examined from slides of normal cultures to detect aneuploidy and 50 cells to detect structural aberrations (Figure 2), i.e. gaps, chromatid and chromosome breaks that were classified according to the criteria suggested by Savage (22) and Carrano and Natarajan (23). All metaphase plates were observed under a fluorescence Nikon Eclipse 80i microscope, captured with a Nikon Sight DS-5M digital camera, transferred to PC and later processed by image analysis software of two cytogeneticists.

The two groups were compared with the following statistical tests: (i) average difference evaluation with Student's *t*-test of gaps, chromatid and chromosome breaks, CAs excluding gaps and aneuploidy; (ii) evaluation of group variance difference using *F* test; (iii) evaluation of intra-group variance using χ^2 test and (iv) evaluation of intra-farm variance using χ^2 test. The aims were to verify whether average differences between groups 1 and 2 were statistically significant and whether the differences were not related to the farms but only to the TH.

Results

At birth, all buffalo calves with TH were alive and lively. The only symptom was ambulatory difficulty due to the malformed limb. Indeed, at necroscopy, they showed no macroscopic lesions incompatible with life.

The following clinical and radiological patterns were observed in the malformed animals: hind limbs amputated, the right amputated off the second tarsus bones and the left amputated off the left off diaphysis tibia (1 female); left hind limb amputated off the proximal epiphysis metatarsus (2 females and 1 male); left hind limb amputated off the third tarsus bones (1 female); left hind limb amputated off the tibia (1 female and 1 male); left hind limb amputated off the distal epiphysis metatarsus (1 female); left hind limb amputated off the first phalanx (1 male); right hind limb amputated off the proximal epiphysis metatarsus (1 male); left hind limb amputated off the proximal epiphysis tibia (1 female) and right hind limb amputated off the proximal epiphysis tibia (2 males). In their malformed limbs, all the animals presented more or less developed outlines of claws (Table I).

Table II shows the percentage of aneuploidy and mean values and standard deviations of chromosome abnormalities in cells of groups 1 and 2 (control). The percentage of aneuploid cells was higher in animals with TH (12.76) than in control animals (7.85). Mean values of gaps are higher in cells of animals with TH (6.62 ± 2.38) than those found in the control (2.86 ± 1.01), and similar results were obtained in chromatid breaks (0.13 ± 0.31 and 0.07 ± 0.06, respectively), chromosome breaks (0.11 ± 0.27 and 0.06 ± 0.13, respectively) and CAs excluding gaps (0.24 ± 0.47 and 0.13 ± 0.18, respectively) (Figure 3).

The *F* values (Table III) reveal that the variances in all studied parameters are independent ($P < 0.001$). Statistical analysis carried out with the *t*-test shows highly significant increases ($P < 0.001$) in the mean number of malformed buffalo calves' gaps, chromatid and chromosome breaks and CAs without gaps compared with that in the control (Table IV).

As for the analysis of intra-group variance carried out with the χ^2 test, only 4% of the variability depends on the TH/healthy condition and 96% on TH. Comparing the animals with TH grouped from farms using the χ^2 test, it may be asserted that the TH condition does not stem from the farm because the variance in farms is 11.75 while among farms it is 0.24, and the general standardized variance is 12.

Discussion

To this day, developmental limb malformations in humans have been linked to genetic and environmental factors, teratogenic agents and drugs or to their combinations (24,25). In the last few years, an increasing number of buffalo breeders in southern Italy have reported the birth of calves affected by TH, the origin of which is still unknown. However, the action of genotoxic factors can be excluded since no pollutants were revealed in environmental analyses (food and soil).

In livestock, several congenital malformations such as amelia (6), polymelia (21) and ectromelia (20) have been associated to genomic instability shown by increased values of micronuclei, CAs and SCEs.

A preliminary study (26) carried out on 10 Mediterranean Italian buffaloes by using the SCE test showed a statistically highly significant difference ($P < 0.001$) in the mean rate of SCE/cell (8.80 ± 3.19 and 6.61 ± 2.73 SCE/cell, respectively, in malformed calves and the control group).

To better characterize this kind of chromosome instability, we applied the CA test on a larger sample (13 buffalo calves with TH), finding that the percentage of aneuploidy and the mean rate of all the types of structural aberrations/cell are higher in animals with TH than in the control and that, according to the *t*-test, these differences are statistically significant ($P < 0.001$, Tables III and IV). To exclude that these differences are related to breeding area and type, we analysed the variance within the animals with TH divided into groups according to the four farms of origin. Our results show that there are no differences, thus indicating that the higher

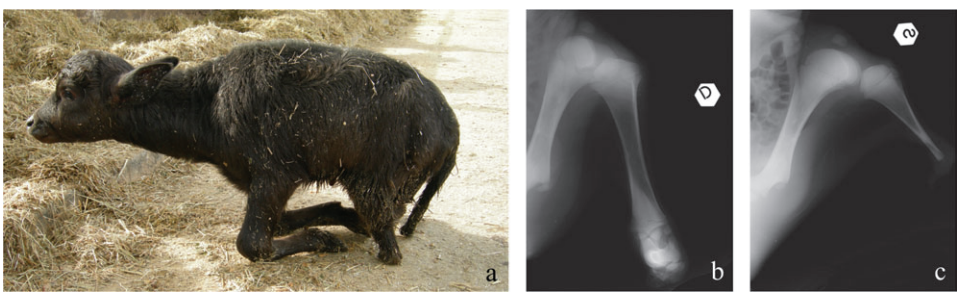

Fig. 1. (**a**) Italian Mediterranean buffalo calf (see Table I, Case 1) with both hind limbs amputed. (**b**) Lateral view of the right hind limb (Case 1) with TH at level of the distal row of the tarsus. (**c**) Lateral view of the left hind limb (Case 1) with TH at level of the diaphysis tibia.

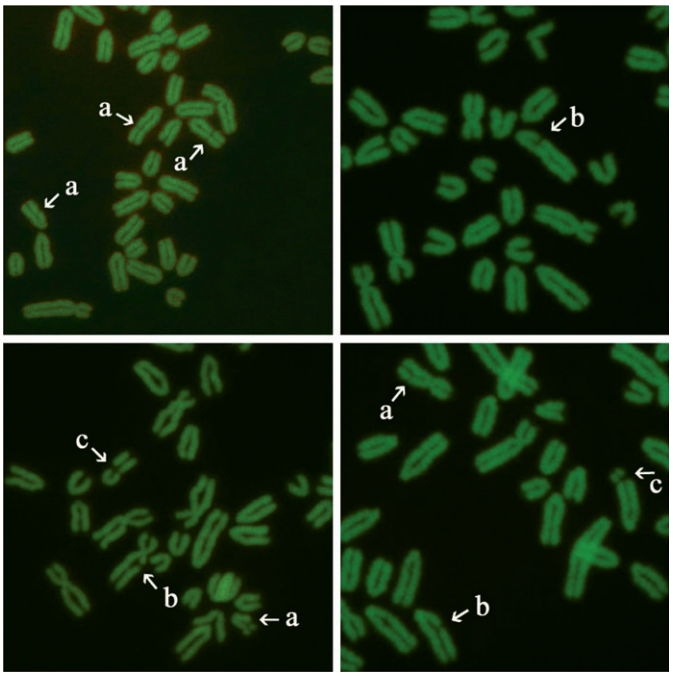

Fig. 2. CAs in buffalo metaphases: a, gaps; b, chromatid breaks; c, chromosome breaks.

Table I. Anatomic descriptions of limb malformation in 13 Mediterranean Italian buffalo calves

Case	Sex	Limb malformation (TH)
1	F	Hind limbs amputated the right off second tarsus bones and the left off diaphysis tibia
2	F	Left hind limb amputated off proximal epiphysis metatarsus
3	M	Left hind limb amputated off proximal epiphysis metatarsus
4	F	Left hind limb amputated off third tarsus bones
5	F	Left hind limb amputated off proximal epiphysis metatarsus
6	F	Left hind limb amputated off proximal epiphysis tibia
7	M	Left hind limb amputated off distal epiphysis tibia
8	M	Right hind limb amputated off proximal epiphysis metatarsus
9	M	Left hind limb amputated off first phalanx
10	F	Left hind limb amputated off distal epiphysis metatarsus
11	F	Left hind limb amputated off proximal epiphysis tibia
12	M	Right hind limb amputated off proximal epiphysis tibia
13	M	Right hind limb amputated off proximal epiphysis tibia

Table II. Percentage of aneuploidy and mean values and standard deviations of chromosome abnormalities in groups 1 and 2 (control) cells

Group	Aneuploidy ($2n < 50$) %	Gaps Mean ± SD	Chromatid breaks Mean ± SD	Chromosome breaks Mean ± SD	CAs excluding gaps Mean ± SD
1	12.76	6.62 ± 2.38	0.13 ± 0.31	0.11 ± 0.27	0.24 ± 0.47
2	7.85	2.86 ± 1.01	0.07 ± 0.06	0.06 ± 0.13	0.13 ± 0.18

SD, standard deviation.

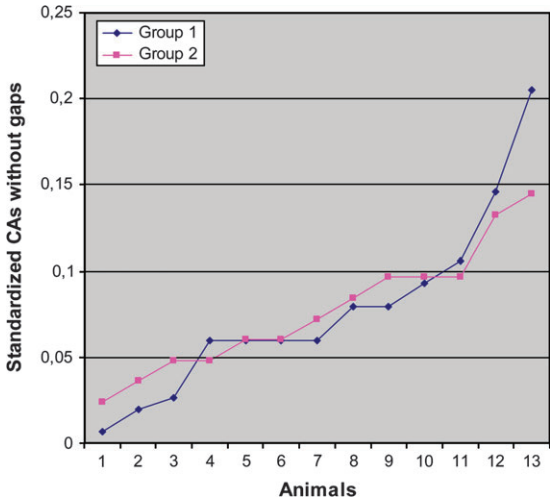

Fig. 3. Distribution of CAs without gaps in group 1 (animals with TH) or group 2 (control animals).

Table III. *F* values of the observed differences in the mean rate of gap/cells, chromatid break/cells, chromosome break/cells and CAs excluding gaps/cells within the group 1–group 2 comparison

Parameter	*F*
Gap/cell	8.19*
Chromatid break/cell	19.08*
Chromosome break/cell	8.67*
CAs excluding gaps/cells	9.16*

*$P < 0.001$.

number of CAs in animals with TH is related only to the congenital malformation.

Comparing the frequency of CAs (excluding gaps) in buffalo with TH (0.24 ± 0.47) with the data of Ahmed *et al*. (15) on genomic instability in buffaloes exposed to industrial (30.8 ± 0.97) and car (31.3 ± 1.83) exhaust emissions of genotoxic agents in Cairo and Shoubra El-Kheima (Egypt), the differences are very high both for the age range of the animals analysed (from few days to 6 months for the buffaloes with TH in the present study and from 3 to 5 years for the exposed animals in the Ahmed study) and for the causes of the genomic instability. Indeed, genotoxic agents determine a higher degree of genomic instability than mutational events.

The mutations that caused the abnormal phenotypes of TH cannot be identified by cytogenetic tests and it will therefore be necessary to apply molecular genetic studies to discover the mutations and the genes involved in this malformation.

In humans, the developmental malformations of the limbs linked to genetic factors are related to mutations of several genes. Recently, WNT3 mutation was found in four consanguineous foetuses affected by tetra-amelia showing that this gene is required at the earliest stages of human limb formation (27), while a partial or a total loss of WNT7A function, due to a variety of mutations, causes less or more severe limb malformations (Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome) (3). Identification of gene mutation involved in TH in Italian Mediterranean buffalo is very difficult since the genes that regulate limb development are not yet well characterized and the buffalo genome has not yet been sequenced.

Our future aims are to verify whether the gene mutations identified in some human limb malformations (such as WNT3, WNT7A and ESCO2) (27–29) are also involved in buffalo TH or if other genes responsible for embryonic limb development are involved (like Shh, Fgfs, BMPs and EN1) (4,30).

Table IV. Significance levels of the observed differences within each comparison (*t*-test)

Mean rate	Group 1–group 2
Gap/cell	45.51*
Chromatid break/cell	5.30*
Chromosome break/cell	5.11*
CAs excluding gaps/cells	6.22*

*$P < 0.001$.

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