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EDITORIAL

Pre-participation screening for the prevention of sudden cardiac death in athletes

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Abstract

Pre-partecipation screening is the systematic practice of medically evaluating large populations of athletes before participation in sport activities for the purpose of identifying abnormalities that could cause disease progression or sudden death. In order to prevent sudden cardiac death (SCD), cardiovascular screening should include a strategy for excluding high-risk subjects from athletic and vigorous exercise. There are two major screening programmes in the world. In the United States competitive athletes are screened by means of family and personal history and physical examination. In Italy there is a mandatory screening for competitive athletes, which includes a resting electrocardiogram (ECG) for the detection of cardiac abnormalities. The most important issue to be addressed is whether a screened subject is really guaranteed that she/he is not suffering from any cardiac disease or at risk for SCD. Conceivably, the introduction of echocardiogram during the pre-participation screening, could be reasonable, despite the discrete sensitivity of ECG, in raising clinical suspicions of severe cardiac alterations predisposing to SCD. It is clear that the cost-benefit ratio per saved lives of the ECG screening is a benchmark of the Public Health policy. On the contrary, the additional introduction of echocardiography in a large population screening programme seems to be too much expansive for the Public Health and for this reason not easily practicable, even if useful and not invasive. Even if we strongly believe that a saved life is more important than any cost-efficacy evaluation, the issue of the economical impact of this approach should be further assessed.

Key words: Sudden cardiac death; Prevention; Athletes; Pre-participation screening; Screening

Core tip: The review underlines the different incidence of sudden cardiac death (SCD) and the reason of this discrepancy as well as the different kind of approach to competitive athletes in Italy and United States. We emphasize the importance of electrocardiogram (ECG) as a simple and economical diagnostic tool, considering the opportunity of implementing the pre-participation screening (PPS) with echocardiogram, in order to detect mild structural cardiac diseases. After a cost-effectiveness analysis we finally suggest an innovative proposal in order to further prevent SCD: a rapid but focused echocardiogram assessment during the first PPS in addition to physical examination and ECG.

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SUDDEN CARDIAC DEATH

The Framingham definition of sudden death (SD) is the most universally used among medical researchers: "SD

is a death that occurs within 1 h of the onset of symptoms". Other definitions are commonly used, for example Maron's one: "SD is defined as a witnessed or unwitnessed natural death occurring unexpectedly within 6 h of a previously normal state of health"[1].

Most of the sport related SDs (almost 90%) occur in subjects who have pre-existing and often clinically silent cardiac abnormalities[2]. In these circumstances the sudden cardiac death (SCD) is defined as "non traumatic, nonviolent, unexpected natural death of cardiac origin occurring within 1 h of the onset of symptoms in a person who does not have a previously recognized cardiovascular condition that would appear fatal"[1].

When considering athletes, sport related SCD occurs during or immediately after physical exercise. Hence, physical efforts are considered an important acute trigger factor (like emotional stress, environmental factors, sympathetic-vagal imbalance, myocardial ischemia, hemodynamic changes) able to interact with a substrate causing life threatening ventricular tachyarrhythmias[3]. The incidence of SCD during sports is quite low and variable among the different studies available in literature. Moreover, a distinction between older athletes (> 35 years) and young athletes (< 35 years) is mandatory when analyzing SCD events. Indeed, in the former group the most important cause of SCD is represented by premature coronary artery disease (CAD)[4,5]; less frequently by acquired valvular diseases and cardiomyopathies (*i.e.*, hypertrophic cardiomyopathy)[6]. The incidence of SCD in this age group ranges from 1:15000 to 1:50000[7-9]. When considering younger athletes, autopsy-based studies and epidemiologic observations often revealed structural cardiac abnormalities such as inheritable cardiomyopathies as well as congenital coronary artery anomalies[10]. Other relatively common causes of SCD in subjects under 35 years of age are represented by: myocarditis, Marfan syndrome, valvular heart diseases, dilated cardiomyopathy, CAD, and myocardial bridge[2,11].

In addition, there is a growing number (from 2% to 10%, up to 31% for Australian authors) of unexplained death, even after autopsy, plausibly due to ionic-channel disorders such as short and long QT syndrome, catecholaminergic polymorphic ventricular tachycardia, or Brugada syndrome[12-14]. Finally, Haïssaguerre *et al*[15] recently reported that "among patients with an history of idiopathic ventricular fibrillation, there is an increased prevalence of early repolarization", opening in this way a "new age"of SCD identification.

The incidence of SCD in young athletes ranges from 2.1/100000 (Italy)[8] to 0.4-0.6 per 100000 athletes per year in the United States[16]. This discrepancy is mainly explained by the different sources of information adopted. Indeed, in the United States available data have been obtained through the review of public media reports and other available electronic resources, which could, obviously, underestimate the true incidence of SCD. On the contrary, in Italy a prospective registry of juvenile SD is regularly updated. In addition, it is necessary to consider other differences that could further justify the observed discrepancies; namely differences of age (older athletes in the Italian series) and gender (larger proportion of females in the United States series). Smaller studies, performed as population based investigations, reported, also in the United States, an incidence of either SCD of young individuals and athletes similar to that reported by Shen *et al*[17] in Italy. Similarly, Maron *et al*[18] in 2009 reported an annual incidence of SCD among United States high-school and college athletes roughly similar to that observed in Italy.

In any case, differences between Italian and United States data, constitute the basis of the debate between two different approaches to pre-participation screening (PPS): (1) American Heart Association (AHA) consensus panel: (medical history and physical examination)[19]; and (2) European Society of Cardiology (ESC) consensus panel (based on Italian PPS): medical history and physical examination plus a 12-lead electrocardiogram (ECG) with interpretation[20].

PPS

Both AHA and ESC recommend and emphasize the weight of ECG screening, but the AHA 2007 update sustains the impracticability of PPS with ECG in the United States because of the lack of logistical and economical resources[21]. Actually, high schools athletes and Olympic athletes, usually undergo, in United States, a screening based on medical history and physical examination, which seems inadequate to prevent SD. This hypothesis is supported by a case record of Maron retrospectively analyzing 134 cases of SD. This study concluded that only 3% of the SD were suspected through medical history and physical examination[2].

The contradiction of this approach has been extensively discussed in the recent literature. The most important issue is that professional athletes, already evaluated during the years and most likely healthy, are more safeguarded when compared to young adolescents at the beginning of their sport activity, when congenital or genetic diseases typically arise[21]. This everlasting dispute, will hardly come to an end since it reflects two different philosophies as well as two different ways of considering Public Health. Notably, in Italy PPS, after the abolition of compulsory military service, is now the fundamental medical screening for apparently healthy youths. Indeed 5 million of athletes repeat the medical evaluation every year since it is compulsory for competitive sport.

PPS, in Italy, is a general medical screening and the cardiovascular system is deeply investigated, since most of the sport related SD is of cardiac origin[10]. It consists in medical history, physical examination, spirometry, urine analysis plus basal and after step test 12-lead ECG[22]. Second level investigations include: echocardiogram, stress test[23], 24 h ECG and ambulatory blood pressure monitoring; all requested in case of clinical and/or electrocardiographic abnormalities. In Italy, all

athletes are evaluated accordingly to the current Cardiologic Protocols for Competitive Sports Qualification. This document indicates guidelines for all the cardiovascular diseases, including high risk early repolarization patterns, isolated left ventricle non-compaction as well as many other doubtful conditions[24].

The huge experience obtained with PPS in Italy, clearly indicates that ECG, characterized by an excellent negative predictive value, when compared with only physical examination, is an essential tool to suspect or diagnose cardiac structural and electric channels pathologies[20,25]. The usefulness of ECG as a preventive tool during PPS, is underlined by one of the most significant studies of Corrado, showing how the annual incidence of SCD in competitive athletes decreased by 89% in Italy, after the introduction of PPS. In particular, SCD cases varied from 3.6/100000 athletes per year during the pre PPS period (1979-1981) to 0.4/100000 athletes per year during the PPS period (1993-2004)[26]. In particular, Corrado *et al*[27] confirmed the efficacy of ECG in the identification of hypertrophic cardiomyopathy. Indeed, in a large population-based study of screening outcomes, the diagnostic power of ECG was similar to that observed in a population-based study in the United States, using echocardiography[28]. Nevertheless, it's comprehensible how difficult it is to identify, despite instrumental evaluation, many mild structural cardiac abnormalities, eventually associated with short and long-time hemodynamic and arrhythmic sequels. This observation gains even more strength when considering children and adolescents since such cardiac abnormalities are still not complicated and for this reason usually clinically silent[28,29].

Unfortunately, even if ECG has to be considered the most reliable and feasible analysis to detect severe cardiomyopathies[10,30,31] as well as ionic channel disorders[32] many asymptomatic pathologies could remain unrecognized through standard PPS. For example, mitral valve prolapse (MVP) and bicuspid aortic valve (BAV), considered among the most common congenital cardiomiopathies in the adult population (0.6% up to 2.4%, in the Framingham heart study, for MVP[33,34] and 0.5%-0.6% for BAV[35]) remain often undiagnosed through PE and ECG. With this regard, it is known that these valvulopaties are frequently complicated by severe dysfunctions requiring cardio surgery[36-38], that more than 50% of BAV evolve to root or proximal ascending aorta dilatation caused by wall vessel structural alterations, with possible spontaneous rupture and dissection[39,40] and that, occasionally, MVP and BAV are the only pathological findings at the autopsy of athletes death suddenly[2,41]. For these reasons, it has been proposed to implement standard PPS with echocardiography in order to allow a risk stratification as well as an adequate follow up and recommendations[42,43]. With this regard, it has to be considered that the overall prevalence of mild cardiac pathologies in asymptomatic subjects has not been entirely investigated.

Steinberger *et al*[44] reported a prevalence of 3.6% of cardiomiopathies among 357 asymptomatic children. More recently, we evaluated, with the introduction of echocardiography during the PPS, a large population (3100 athletes) of active, asymptomatic, apparently healthy children and adolescents, finding a prevalence of 1.8% of previously unrecognized cardiomiopathies[45]. Taken together, these observations may suggest that the introduction of echocardiogram during the PPS, could be reasonable, despite the discrete sensitivity of ECG, in raising clinical suspicions of severe cardiac alterations predisposing to SCD. Obviously, a 2.7% (our study) and 3.6% (Steinberg' study) of prevalence of structural cardiac disease cannot justify the execution of echocardiogram every year in competitive athletes. Nonetheless, it has to be underlined the usefulness of echocardiogram in association with ECG in occasion of the first visit since the early diagnosis and subsequent reparation of congenital lesions, reduce the risk of future hemodynamic and arrhythmic complications.

It is well known that ECG abnormalities are commonly found both in patients affected by potentially life-threatening congenital cardiac disorders and healthy highly trained subjects as a result of the athletes' hearth modifications. In the latter situation, it is usually necessary to perform a differential diagnosis by means of an echocardiography study. Sometimes, this analytical approach is not enough to reach a precise diagnosis and further evaluations are commonly requested. This situation is surely depressing for the athletes since they have to suspend their competitive or training programmes with potential detrimental effects on their competitive season. However, sports physicians have the legal duty to reach a definitive and correct diagnosis with any diagnostic tool available in order to ensure that the subject does not run unjustified and excessive risks. In case of uncertainty, sports physicians have the duty of rejecting sports eligibility[10]. On the contrary, it has to be remembered that a normal ECG have an high predictive negative value (96%)[46].

The last issue to be analyzed is the cost-effectiveness of this approach. Despite the unquestionable usefulness of a wide scale screening performed with echocardiogram on a large healthy population, it is obviously necessary to analyze the costs for Public Health. Recent data from the "National Centre for Health Statistics"[47] sustained the efficacy and the feasibility of the PPS based on medical history, PE and basal ECG, both when considering the cost-benefit ratio and the saved lives. In literature, it has been described that ECG has a more favorable cost-effectiveness ratio per life saved among high school athletes when compared to the adoption of only medical history and PE[48]. The same conclusion was described by Wheeler *et al*[49] (cost effectiveness ratio of $ 42900 per life year). In conclusion, it is clear that the cost-benefit ratio per saved lives of the ECG screening is below $ 50000 which is a benchmark of the Public Health policy[48-51].

Despite these observations, some United States au-

Table 1 Italian and United States pre-participation screening strategy

Italian pre-participation screening		United States pre-participation screening	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
Rest ECG screening power (ionic-channel disorders, arrhythmias and cardiomyopathies)	Higher cost	Lower cost	Low diagnostic power in the detection of silent cardiovascular diseases
Post exercise ECG diagnosis (arrhytmias, stress-induced myocardial ischemia)	More complicated logistic	Easy logistic	Impossibility to detect post exercise ECG alterations
Spyrometry (pulmonary diseases)	More false positive	Less false positive	Only clinical diagnosis of pulmonary diseases
Urine analysis (diabete, proteinuria, kidney, liver and urogenital infectious diseases)	Possible psychological ramification of the screening	Easy feasibility	Athletes can compete without permission (PPS is not mandatory)
In Italy athletes health is protected by law (PPS is mandatory)			Many cardiovascular disorders cannot be detected by physical examination

ECG: Electrocardiogram; PPS: Pre-participation.

Table 2 Cost-efficacy trials

Studies	Considerations
Cost effectiveness analysis of screening of high school athletes for risk of sudden cardiac death[48]	A more favourable cost-effectiveness ratio of ECG when compared to medical history collection and physical examination or 2D echocardiograph
Cost-effectiveness of preparticipation screening for prevention of sudden cardiac death in young athletes[49]	Screening young athletes with 12-lead ECG plus cardiovascular-focused history and physical examination may be cost-effective
Usefulness and cost effectiveness of cardiovascular screening of young adolescents[50]	The cost of this screening system was lower when compared to the United States model
An electrocardiogram should not be included in routine prepartecipation screening of young athletes[52]	Implementing PPS with ECG could be too much expansive in the United States, in consideration of the enormous number of competitive high school and college athletes
Preventing sudden death of athletes with electrocardiographic screening: What is the absolute benefit and how much will it cost?[53]	The Italian strategy of ECG screening in the United States would result in enormous costs per life saved

ECG: Electrocardiogram; PPS: Pre-participation.

thors still discourage the adoption of ECG in routine PPS of young athletes since its cost seems to be excessive for the public health[52,53] (Tables 1 and 2).

Another viewpoint has been provided by the Israeli experience. Indeed, Israeli Sport Law implemented PPS in 1997. As reported in literature, they did not find any difference in the incidence of SCD in athletes following the introduction of PPS. For this reason they concluded that PPS is neither useful nor cost effective[54]. This divergence could be explained by the result of a recent Asian study illustrating the main causes of SCD in Israel: Marfan's syndrome, anomalous coronary arteries, catecholamine related arrhythmias and commotion cordis. All these pathologies are much more difficult to be detected by resting ECG when compared ti hypertrophic cardiomyopathy, dilated cardiomyopathy, valvular heart disease, ARVD, long QT syndrome and Brugada's syndrome, all representing the most common cause of SCD in Europe and United States[55].

Undoubtedly, echocardiogram seems to be too much expansive for the Public Health and for this reason not easily practicable, even if useful and not invasive. Partial solutions of this issue have been proposed by Weidenbener *et al*[56] with the inclusion of a single-view parasternal long and short-axis two-dimensional screening echocardiogram at an average cost of $7.34 per examination. The effectiveness of this approach has been confirmed during PPS[57] as well as for the follow up of other cardiovascular diseases such as hypertension in order to evaluate left ventricular hypertrophy[58,59].

Even if we strongly believe that a saved life is more important of any cost-efficacy evaluation, the issue of the economical impact of this approach should be further assessed.

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BRIEF ARTICLE

Confidence limit calculation for antidotal potency ratio derived from lethal dose 50

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Abstract

AIM: To describe confidence interval calculation for antidotal potency ratios using bootstrap method.

METHODS: We can easily adapt the nonparametric bootstrap method which was invented by Efron to construct confidence intervals in such situations like this. The bootstrap method is a resampling method in which the bootstrap samples are obtained by resampling from the original sample.

RESULTS: The described confidence interval calculation using bootstrap method does not require the sampling distribution antidotal potency ratio. This can serve as a substantial help for toxicologists, who are directed to employ the Dixon up-and-down method with the application of lower number of animals to determine lethal dose 50 values for characterizing the investigated toxic molecules and eventually for characterizing the antidotal protections by the test antidotal systems.

CONCLUSION: The described method can serve as a useful tool in various other applications. Simplicity of the method makes it easier to do the calculation using most of the programming software packages.

Key words: Up-and-down method; Confidence limit; Potency ratio, Bootstrapping

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INTRODUCTION

To characterize toxic effects of poisons and overdosed drugs, acute toxicity testing methods were developed in the beginning of the 19th century. Trevan[1] first wrote up the concept of lethal dose 50 (LD_{50}) (medium lethal dose) in 1927. He also indicated that LD_{50} is not a biological constant, its precision depends on many factors (*e.g.*, number of animals used, sex, species, strain, age, diet, general health condition, route of administration, stress, formulation, intra and inter laboratory variations, *etc.*). To express acute toxicity, LD_{50} is a good tool, and many government agencies still rely on these data. Many methods have been developed to characterize the toxic effects (acute toxicity) of chemicals, and expressed as LD_{50} values and its 95% confidence limit and the slope of the probit line, *e.g.*, Litchfield and Wilcoxon[2], Bliss[3], Holland *et al*[4]. Up until the 90s, the Litchfield and Wilcoxon[2] method was one of the most frequently used tool for toxicologists to characterize acute toxicity, and the *in vivo* antidotal efficacy of various antidotal systems.

An example, Pei *et al*[5], analyzed data for LD_{50} values of paraoxon that is an organophosphorus (OP) type nerve agent, in the presence of various antidotal systems by the method of Litchfield and Wilcoxon, as adapted to

a computer program PHARM/PCS version 4.2. by Bergol'ts *et al*[6]. The antidotal potency ratios (APRs) derived from the dose-response curves of paraoxon were used to express the *in vivo* efficacy of various OP antidotal systems to antagonize the lethal effects of paraoxon (APR = LD_{50} of paraoxon antagonized/LD_{50} of paraoxon unantagonized). Tests for the parallelism of the dose-effect curves were done, and all statistical procedures were performed at the 95% confidence level. The authors used six groups of animals, 8 animals per groups (48 animals) for each LD_{50} value.

Since the Litchfield-Wilcoxon method requires a large number (40-50) of animals, efforts were done to introduce other LD_{50} determinations with a lesser number of animals. The up-and-down methods by Dixon[7], Bruce[8] can provide adequate estimation of LD_{50} and approximation of the 95% confidence interval by using as few as 6-9 animals. When this method was compared with the traditional Litchfield-Wilcoxon method, excellent agreement was obtained for all the 10 molecules tested.

Another example: Petrikovics *et al*[9], determined LD_{50} values for paraoxon by the method of Dixon[6], and 95% confidence limit was estimated by the method of Bruce[10]. For each experiment, 6-10 animals were used. The LD_{50} values were calculated from the equation of *Log (*LD_{50}*) = log (dose final) + k log (d)* where *dose final* is the final dose administered, *k* is the tabular value from the table, and *d* is the interval between doses. APRs were expressed as a simple number (without confidence limit). APR = mean LD_{50} of paraoxon antagonized/mean LD_{50} of paraoxon unantagonized.

Another example: Petrikovics *et al*[11], determined LD_{50} for cyanide by the up-and-down method of Dixon[7]. This method requires settings for the starting doses and the stage distances (dose difference between doses) for each test system. The software (based on "Implementation of Dixon and Massey UPD", Introduction to statistical Analysis, 1983, pp.434-438) provides information for the next dose for each stage, based on the mortality results for the given stage. The log dose difference of 0.1 was set up based on the earlier studies with cyanide (Petrikovics *et al*[12], where the LD_{50} values were determined by the classic Litchfield-Wilcoxon[2] method. For each LD_{50} values 10-18 were used. LD_{50} values were expressed as average ± 95% confidence limit by the software. APRs were expressed as a ratio of average LD_{50} of cyanide with antidotes and average LD_{50} of cyanide without antidotes. Again, confidence limits for APR were not expressed.

MATERIALS AND METHODS

In a situation like this where the distribution of the ratio is unknown, it is difficult to calculate the confidence intervals using classical methods. However, we can easily adapt the nonparametric bootstrap method which was invented by Efron[13] to construct confidence intervals in such situations like this. The bootstrap method is a resampling method in which the bootstrap samples are obtained by resampling from the original sample. A comprehensive coverage of the bootstrap method can be found in Efron and Tibshirani[14]. Chernick[15], Shao and Tu[16], Davison and Hinkley[17], Manly[18] and Hayden[19] are also useful references.

There are several ways of calculating bootstrap confidence intervals. Briefly, one way of calculating the bootstrap confidence interval for APR given below:

To assume that the data set is coming from two samples which we call sample 1 and sample 2 to calculate the APR.

(1) Obtain a bootstrap sample $X^* = (X_1^*, X_2^*,\ldots\ldots, X_{n1}^*)$ from the original sample 1 $X = (X_1, X_2,\ldots\ldots, X_{n1})$.

(2) Calculate logLD_{50} dose estimate using

$$LD_1^* = \frac{\Sigma X_i^*}{n_1} + \frac{d}{n_1}(A_1 + C_1)$$

[page 389 Dixon (1969)];

(3) Obtain a bootstrap sample $Y^* = (Y_1^*, Y_2^*,\ldots\ldots, Y_{n1}^*)$ from the original sample 2 $Y = (Y_1, Y_2,\ldots\ldots, Y_{n1})$;

(4) Calculate logLD_{50} dose estimate using,

$$LD_2^* = \frac{\Sigma Y_i^*}{n_2} + \frac{d}{n_2}(A_2 + C_2)$$

(5) Calculate the ratio,

$$APR^* = \frac{10^{LD_1^*}}{10^{LD_2^*}}.$$

(as the values are in log base 10);

(6) Repeat step 1 through step 5, a large number of times (B = 1000) to get a list of values;

(7) Find the quantiles $APR_{(\alpha/2)}$ and $APR_{(1-\alpha/2)}$ for the list of *B* ratio values. ($APR_{\alpha/2}$, $APR_{1-\alpha/2}$) is the 100 (1 - α)% confidence interval for the ratio. This confidence interval is usually called percentile bootstrap confidence interval.

RESULTS

We illustrate the method for LD_{50} ratio for the following two experiments (Figure 1). Figure 2 shows the histogram of the bootstrap distribution of the APR. Quantiles of this distribution are used to derive the relevant confidence limits. In our illustration here we use the 95% confidence limit. LD_{50} dose estimate for the first experiment is 7.834 and the LD_{50} dose estimate for the second experiment is 23.812. This gives the APR to be 0.32897. Therefore, the lower confidence limit, which is the 2.5th percentile of the bootstrap distribution is 0.25821 and the upper confidence limit which is the 97.5th percentile of the bootstrap distribution is 0.41714.

DISCUSSION

We used a simple method to construct the confidence interval for calculating APR derived from two LD_{50}. The described nonparametric bootstrap method to determine confidence intervals can easily be constructed even in situations where the distribution of the ratio is un-

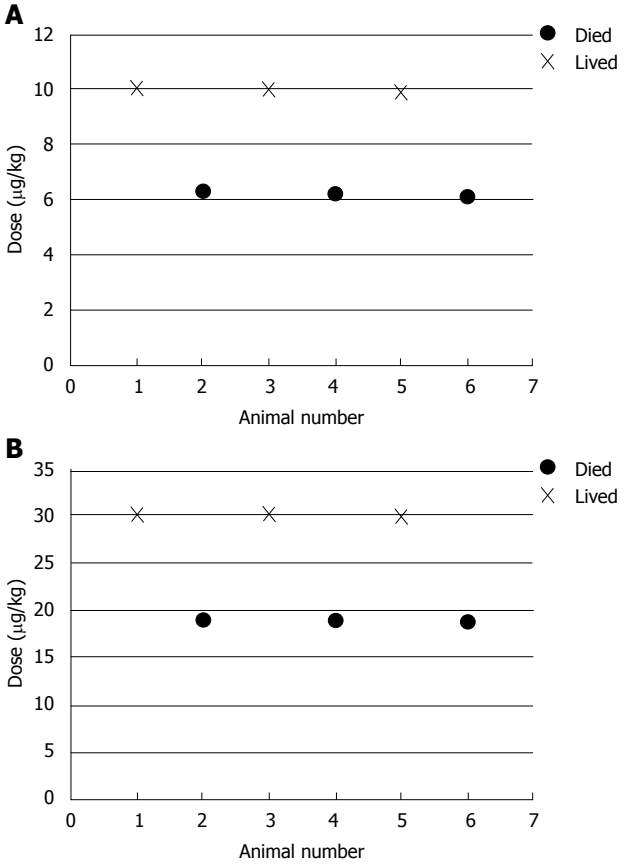

Figure 1 Graph of the dose and the outcome (lived or died) for the first (A) and second (B) experiment.

known. This presentation describes a calculation of the bootstrap confidence interval for APR. This can serve as a substantial improvement for toxicologists, who are directed to employ the Dixon up-and-down method with the application of lower number of animals to determine LD_{50} values for characterizing the investigated toxic molecules and eventually for characterizing the antidotal protections by the test antidotal systems. The described method can serve as a useful tool in various other applications. Simplicity of the method makes it easier to do the calculation using most of the programming software packages.

COMMENTS

Background

To characterize toxic effects of poisons and overdosed drugs, acute toxicity testing methods were developed in the beginning of the 19th century. To express acute toxicity, lethal dose 50 (LD_{50}) is a good tool, and many government agencies still rely on these data. Many methods have been developed to characterize the toxic effects (acute toxicity) of chemicals, and expressed as LD_{50} values and its 95% confidence limit and the slope of the probit line. To characterize antidotal efficacy of a given antidotal system, antidotal potency ratios (APRs) are calculated, that is the ratio of the LD_{50} of the toxic chemical with the test antidotal system and the LD_{50} of the toxic chemicals without any antidote(s) (control). The higher is the APR, the better is the antidotal system.

Research frontiers

When applying the classic Litchfield-Wilcoxon method for LD_{50} determination, a large number of animals (6-8 groups of animals, 6-8 animal/group = 36-64) are needed. To reduce the number of animals, new methods were developed, and the Dixon up-and-down method has become popular with its lower number of animal needed (8-18 animals/LD_{50}). However, when the Litchfield-Wilcoxon method was adapted to a computer program PHARM/PCS version 4.2. by Tallarida and Murray, the APR was automatically expressed with 95% confidence limits by the software. There is a need for the 95% confidence limit determination with the Dixon up-and-down method when expressing APR values. Although Bruce provided adequate estimation for it, this article introduces a more practical tool for filling this gap.

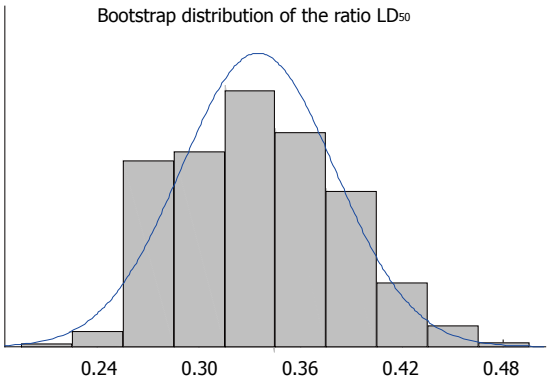

Figure 2 Bootstrap distribution of antidotal potency ratio. LD_{50}: Lethal dose 50.

Innovations and breakthroughs

Previous methods to characterize acute toxicity and/or determining antidotal efficacy for antidotal systems needed to be transformed in order to (1) reduce the number of animals used for LD_{50} determination (2) calculate 95% confidence limits for APR with lower number of animals used. This article can serve as a substantial help for toxicologists, who are directed to employ the Dixon up-and-down method with the application of lower number of animals to determine LD_{50} values for characterizing the investigated toxic molecules and eventually for characterizing the antidotal protections by the test antidotal systems.

Applications

The described method can serve as a useful tool in various other applications. Simplicity of the method makes it easier to do the calculation using most of the programming software packages. Authors used a simple MATLAB code to illustrate the confidence interval for the given example.

Terminology

LD_{50} is the dose that kills 50% of the tested animal population. APR = LD_{50} of the toxic chemicals in the presence of the test antidotal system(s)/LD_{50} of the toxic chemical without any antidote(s) (control). APR is use to express in vivo efficacy for antidotal systems. Bootstrap method is a standard technique in which we take simple random samples with replacement from the original sample. With this, overlapping samples is permissible in this technique. Strength of the paper is the application of the bootstrap method to calculate a confidence interval for the LD_{50} ratio. Validation of the method proven practically and theoretically in the literature.

Peer review

This is a good practical method to express 95% confidence limits for APR derived from the Dixon up-and-down method.

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BRIEF ARTICLE

Methods for the extraction and RNA profiling of exosomes

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Abstract

AIM: To develop protocols for isolation of exosomes and characterization of their RNA content.

METHODS: Exosomes were extracted from HeLa cell culture media and human blood serum using the Total exosome isolation (from cell culture media) reagent, and Total exosome isolation (from serum) reagent respectively. Identity and purity of the exosomes was confirmed by Nanosight® analysis, electron microscopy, and Western blots for CD63 marker. Exosomal RNA cargo was recovered with the Total exosome RNA and protein isolation kit. Finally, RNA was profiled using Bioanalyzer and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) methodology.

RESULTS: Here we describe a novel approach for robust and scalable isolation of exosomes from cell culture media and serum, with subsequent isolation and analysis of RNA residing within these vesicles. The isolation procedure is completed in a fraction of the time, compared to the current standard protocols utilizing ultracentrifugation, and allows to recover fully intact exosomes in higher yields. Exosomes were found to contain a very diverse RNA cargo, primarily short sequences 20-200 nt (such as miRNA and fragments of mRNA), however longer RNA species were detected as well, including full-length 18S and 28S rRNA.

CONCLUSION: We have successfully developed a set of reagents and a workflow allowing fast and efficient extraction of exosomes, followed by isolation of RNA and its analysis by qRT-PCR and other techniques.

Key words: Exosomes; Microvesicles; Cell culture media; Serum; RNA; Quantitative reverse transcription-polymerase chain reaction; Sequencing

Core tip: Exosomes are small vesicles (30-150 nm) perceived to be carriers of the unique effector or signaling macromolecules (miRNA, ncRNA, mRNA and protein) between very specific cells within our body. The spectrum of current scientific interests ranges from studying the functions and pathways of exosomes to utilizing them in diagnostics and therapeutics development. Here we describe a complete exosome workflow solution: fast and efficient isolation of exosomes; extraction of their cargo; characterization of exosomal RNA content using quantitative reverse transcription-polymerase chain reaction and other techniques.

Zeringer E, Li M, Barta T, Schageman J, Pedersen KW, Neurauter A, Magdaleno S, Setterquist R, Vlassov AV. Methods for the extraction and RNA profiling of exosomes. *World J Methodol* 2013; 3(1): 11-18 Available from: URL: http://www.wjgnet.com/2222-0682/full/v3/i1/11.htm DOI: http://dx.doi.org/10.5662/wjm.v3.i1.11

INTRODUCTION

Cells are known to secrete a large variety of vesicles, macromolecular complexes, and smaller molecules like

salts and cofactors, into the extracellular space. The types of vesicles secreted are diverse and depend on the origin of the cells and their current state, for example, transformed, differentiated, stimulated, or stressed. Exosomes are a type of microvesicle, 30-150 nm in size, that have received increased attention over the past decade[1-5]. Exosomes are secreted by all cell types in culture, and also found naturally in body fluids including blood, saliva, urine, and breast milk, in very high numbers (10^8-10^{11} per mL)[6,7]. Depending on the cell/tissue of origin, many different roles and functions have been attributed to exosomes: Facilitators of the immune response[8], antigen presentation[6], programmed cell death, angiogenesis, inflammation, coagulation[9], morphogen transporters in the creation of polarity during development and differentiation[4], and mediation of nontargeted effect of ionizing radiation[10]. Recent studies have demonstrated that exosomes are not only specifically targeted to recipient cells to exchange proteins and lipids or to trigger downstream signaling events, but also to deliver specific nucleic acid cargo for cell communication purposes. Valadi *et al*[11] demonstrated that MC/9 and human mast cell line-1 mast cells secrete exosomes that contain mRNA from approximately 1300 genes and small RNAs, including 121 unique microRNAs. The transfer of exosomes to a donor cell showed that at least some mRNAs were full-length, as they were translated in the recipient cell. Glioblastoma cells also secrete exosomes and microvesicles containing mRNA, miRNA and angiogenic proteins[12] - when taken up by host human brain microvascular endothelial cells, mRNA molecules were translated and tubule formation by the target endothelial cells was stimulated. The spread of oncogenes by exosomes and microvesicles secreted by tumor cells has also been reported[13]. Exosomes seem to play a crucial role in spreading pathogens such as prions and viruses from one cell to another[14-16]. Interest towards exosomes, from their function in the body to more practical applications, such as use in diagnostics and therapeutics development, has grown exponentially in the last few years[17-19].

Critical to further our understanding of exosomes, is the development of reagents, tools and protocols for their isolation, characterization and analysis of their RNA and protein contents. Several reports have been published to date, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and next gen sequencing for initial characterization of the RNA content of exosomes derived from human acute monocytic leukemia cell line, human umbilical vein endothelial cells, dendritic cells and human embryonic stem cell-derived mesenchymal stroma cells, as well as serum, saliva, placenta and breast milk[14,20-22]. All these studies utilize ultracentrifugation protocols[23] which are proven methods, producing clean exosome preparations. However, the ultracentrifugation approach has numerous drawbacks: the method is highly labor intensive and time consuming (up to two days per preparation, for a protocol with sucrose gradient); one can not process more than 6 samples at a time (due to rotor limitation); it requires a large amount of starting material; exosome yields are typically low; extensive training for personnel is needed; and the method overall is not very reliable.

Here we describe a novel approach for fast and efficient isolation of exosomes from cell culture media and blood serum, followed by recovery of RNA cargo and its analysis by qRT-PCR. The procedure is completed in a fraction of the time, compared to the current standard protocols utilizing ultracentrifugation, and allows to recover fully intact exosomes in higher yields.

MATERIALS AND METHODS

Extraction of exosomes from serum and cell media using Total exosome isolation reagents

Cell culture media: Fresh cell media was harvested from HeLa cells, grown initially in the presence of 10% fetal bovine serum (FBS), and [after two phosphate buffered saline (PBS) washes] without FBS for the last 12 h. The cell media samples were then centrifuged at 2000 *g* for 30 min to remove cell debris. The supernatant containing the cell-free cell media was transferred to a fresh container and held on ice until use. Next, each sample was combined with 1/2th volume of Total exosome isolation (from cell culture media) reagent (Invitrogen) and mixed well by vortexing until a homogenous solution was formed. The samples were incubated at 4 ℃ overnight, then centrifuged at 4 ℃ at 10000 *g* for 1 h. The supernatant was aspirated and discarded, and the exosome pellet was resuspended in PBS buffer, then stored at 4 ℃ short term (1-7 d) and -20 ℃ long term.

Human blood serum: Frozen serum samples from different donors were thawed in a water bath at room temperature until samples were completely liquid, then centrifuged at 2000 *g* for 30 min to remove any cellular debris. The supernatant containing the cell-free serum was transferred to a fresh container and briefly held on ice until use. Next, each serum sample was combined with 1/5th volume of Total exosome isolation (from serum) reagent (Invitrogen) and then mixed well by vortexing until a homogenous solution was formed. The samples were incubated at 4 ℃ for 30 min, then centrifuged at room temperature at 10000 *g* for 10 min. The supernatant was aspirated and discarded, and the exosome pellet was resuspended in PBS buffer, then stored at 4 ℃ short term (1-7 d) and -20 ℃ for long term.

Isolation of exosomes from serum and cell media using ultracentrifugation protocols

Cell culture media: Using the same HeLa cell media that was prepared for the extraction of exosomes using the Total exosome isolation reagent, above, exosomes were isolated according to the differential ultracentrifugation Basic Protocol 1 as described by Théry *et al*[23]. Briefly, HeLa cell media was centrifuged at 4 ℃ at 2000 *g* for 10 min and then 10000 *g* for 30 min to produce a cell-

free conditioned medium. The pooled media was divided equally into each of 6 polyallomer tubes and centrifuged at 4 ℃ at 100000 *g* for 70 min. The subsequent pellet was re-suspended and washed with PBS followed by a second 100000 *g* centrifugation. A low volume of PBS was used to re-suspend the washed pellets and then they were combined in pairs to result in three concentrated exosomes samples. These samples were then processed by isopycnic centrifugation using continuous sucrose gradients. The gradients were prepared using solutions of 0.25 mol/L and 2 mol/L sucrose in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.3 as described by Abe *et al*[24].

Once prepared, the sucrose gradients were overlaid with one of the three concentrated exosome samples and then centrifuged at 4 ℃ at 110000 *g* for 16 h. Immediately after centrifugation, each of the gradients was fractionated using a peristaltic pump and fraction collector and each fraction was measured on a refractometer to identify those that contain exosomes based on the density range of 1.13-1.19 g/mL as defined by Record *et al*[25]. The resulting set of fractions were pooled within the respective sample and diluted 3-fold with 20 mmol/L HEPES, pH 7.3 then centrifuged in polyallomer konical™ tubes at 4 ℃ at 110000 *g* for 1 h to produce a final pellet consisting of purified exosome that was re-suspended in 200 μL PBS.

Human blood serum: Using the same human serum that was thawed for the extraction of exosomes using the Total exosome isolation reagent, above, exosomes were isolated according to the differential ultracentrifuge Basic Protocol 2 as described by Théry *et al*[23]. Briefly, serum was diluted with an equal volume of PBS and gently mixed until homogenous. Then the mixture was centrifuged at 4 ℃ at 2000 *g* for 30 min and 12000 *g* for 45 min to produce a cell-free serum. The pooled serum was divided equally into each of the 6 polyallomer tubes and centrifuged at 4 ℃ at 110000 g for 2 h. The subsequent pellet was re-suspended and washed twice with PBS followed each time by centrifugation at 4 ℃ at 110000 *g* for 70 min. A low volume of PBS was used to re-suspend the washed pellets, resulting in three concentrated exosomes samples.

Western blot analysis

Exosome samples isolated from cell media or blood serum (1-5 μL) were mixed with 2 × non-reducing Tris-glycine SDS sample buffer (Novex), then heated at 75 ℃ for 5 min and loaded onto a 1.5 mm × 15 mm well 4%-20% Tris-Glycine gel (Novex). Benchmark prestained protein ladder (Invitrogen) was added to one well as a control to monitor the molecular weight of the protein samples. The gel was run under denaturing conditions at 150 V for 1.5 h then transferred to a membrane using the iBlot instrument (Life Technologies). After transfer, the membranes were processed on the BenchPro 4100 (Life Technologies) with CD63 antibody diluted 100 μg × to 20 mL (Abcam). The WesternBreeze Chemiluminescence kit was utilized to label the membrane. Membranes were then exposed to X-ray film for 1-10 min and the film was analyzed.

RNA isolation

The Total exosome RNA and protein isolation kit (Invitrogen) was utilized for recovery of RNA from both the concentrated exosome samples (reagent and ultracentrifugation) and control samples for each sample type - HeLa cell pellets (1×10^6 cells/pellet) for the HeLa cell culture and cell-free serum for the serum samples. Two hundred microlitre of each sample (brought up to volume with PBS if necessary) was combined with 205 μL of 2× denaturing solution, vortexed to lyse, and then incubated on ice for 5 min. After incubation, 410 μL of Acid-Phenol: Chloroform was added to the mixture and vortexed for 30-60 s to mix. Samples were then centrifuged for 5 min at 10000 *g* at room temperature to separate the mixture into aqueous and organic phases. Once centrifugation was complete, the aqueous (upper) phase was carefully removed without disturbing the lower phase or the interphase, and transferred to a fresh tube. One point twenty-five volumes of 100% EtOH was added to the aqueous phase for each sample then vortexed to mix. About 700 μL of volume was placed onto spin column in a collection tube then spun at 10000 *g* for 15 s to move the sample through the filter cartridge. Samples were then washed once with 700 μL Wash Solution 1 × and 2 × with 500 μL wash solution 2/3 (centrifuged at 10000 *g* for 15 s for each wash). After washing, filter was dried by spinning for an additional 1 min at 10000 *g*. The filter cartridge was transferred into a fresh collection tube and 50 μL of preheated (95 ℃) nuclease-free water was applied to the center of the filter. Samples were centrifuged for 30 s at 10000 *g* to recover the RNA, then a second 50 μL volume of preheated (95 ℃) nuclease-free water was applied to the center of the filter and centrifuged for 30 s at 10000 *g*. After the second spin, the eluate containing the RNA was collected and stored at -20 ℃. A DNase treatment was performed on RNA extracted from the HeLa cell pellet using the DNase-free kit (Ambion) to remove any contaminating DNA. DNase treatment was not performed on other samples as they had a much smaller sample input. After treatment, the sample was diluted to 2 ng/μL and 1 μL of each RNA sample was analyzed on the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Pico kit (Series Ⅱ) to determine the mass of RNA going into downstream analysis.

Reverse transcription and quantitative real-time PCR analysis of the RNA sequences isolated from exosomes

Reverse Transcription (RT) Master Mixes were prepared for each sample using either the High Capacity cDNA Reverse Transcription kit and protocol with random primers for mRNA or the TaqMan® MicroRNA Reverse Transcription Kit reagents and protocol (Applied Biosystems) with gene specific RT primers for five miRNA

targets (let7e, miR26a, miR16, miR24 and miR451). Ten microlitre of the RT Master Mix was added to corresponding wells in a 96-well plate, and 5 μL of each sample was added to the master mix. Plates were covered with adhesive (non-optical) cover and spun down to remove air bubbles, and then placed into a 9700 thermocycler and incubated as follows: for mRNA - 25 ℃ for 10 min; 37 ℃ for 120 min; and 85 ℃ for 5 min; for miRNA - 4 ℃ for 5 min; 16 ℃ for 30 min; 42 ℃ for 30 min; and 85 ℃ for 5 min. Reactions were kept at 4 ℃ until use.

qPCR master mixes were prepared for each of five microRNAs (let7e, miR26a, miR16, miR24 and miR451) and two mRNAs [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S] by combining 5 μL of AB Universal PCR Master Mix Ⅱ, 2.5 μL; of nuclease-free water, and 0.5 μL of the 20 × Taqman Assay. After mixing, 8 μL of each master mix was placed into wells in a 384-well plate (enough for triplicate reactions for each isolation replicate). Two microlitre of each RT reaction was added in triplicate to the master mix of each target and the plates were sealed with optical adhesive cover. Plates were spun down to remove air bubbles then placed into a 7900HT instrument and run using the following thermocycler protocol: 95 ℃ for 10 min; (95 ℃ for 15 s; 60 ℃ for 60 s) 40 cycles. Once the run was complete, automatic Ct analysis was performed with SDSv2.3 software and average and standard deviation was calculated for each set of isolations and qPCR reactions for each target.

RESULTS

Extraction of exosomes from cell media and serum

Isolation of exosomes from cell culture media and body fluids is presently a tedious, non-specific, and difficult process. The widely used approach is based on ultracentrifugation in combination with sucrose density gradients or sucrose cushions to float the relatively low-density exosomes away from other vesicles and particles[23]. The protocols range in time from 8 to 30 h and require an ultracentrifuge and extensive training to ensure successful isolation of exosomes.

As a way to simplify and shorten exosome isolation, we developed two Total exosome isolation reagents that enable straightforward and reliable concentration of intact exosomes from cell culture media and blood serum samples. By tying up water molecules, the reagents force less-soluble components, such as vesicles, out of solution. When the reagent is added to the biological sample, and solution is incubated at 4 ℃, the precipitated exosomes can be recovered by standard centrifugation at 10000 *g*. The pellet is then resuspended in PBS or similar buffer and exosomes are ready for downstream endpoint analysis or biological studies on their pathways, functions and trafficking.

We extracted exosomes from HeLa cell culture media and blood serum samples (derived from healthy human donors) using the Total exosome isolation reagents as well as the ultracentrifugation procedure[23], for comparison purposes. Sizing and quantification of exosomes was performed with the NanoSight® LM10 instrument, following the manufacturer's protocol. This instrument uses a laser light source to illuminate nano-scale particles (10-1000 nm) which are seen as individual point-scatters moving under Brownian motion. The paths of the point scatters, or particles, are calculated over time to determine their velocity which can be used to calculate their size independent of density. The image analysis NTA software compiles this information and allows one to automatically track the size and number of the nanoparticles. Results are shown in Figure 1A and B for HeLa cell culture media, and Figure 2A and B for serum. The reagent method recovered a significant number of nanovesicles, in comparable or higher yields *vs* the ultracentrifugation procedures; All nanovesicles were smaller than 300 nm, most of them being in the typical exosome size range of 30-150 nm. Similar results were obtained for cell media derived from THP-1 and Jurkat cell lines, 1-10 mL sample volume input (data not shown).

Samples were next analyzed by Western blots with antibody specific to CD63 - a well characterized exosomal marker[11,20,23]. Results are shown in Figure 1C for HeLa cell culture media and Figure 2C for serum - confirming that clean exosome populations were recovered with both protocols. CD9, TSG101, Annexin Ⅱ exosomal markers were also confirmed by Western blots (data not shown).

To further characterize samples obtained with the reagent, electron microscopy analysis was performed. Figure 3A shows a representative image of the unlabeled exosome, Figure 3B shows exosome immunolabeled with anti-CD63 antibodies followed by 10 nm protein A gold nanoparticles, and Figure 3C shows exosome immunolabeled with anti-CD81 antibodies followed by 10 nm protein A gold nanoparticles. The exosomes recovered with the reagent have typical appearance and size (about 100 nm[23]), and immunolabeling with anti-CD81 and anti-CD63 antibodies, which are well known exosomal markers[20,23], was very efficient confirming that the nanovesicles recovered with the reagent are exosomes.

RNA isolation and analysis by Agilent 2100 Bioanalyzer and qRT-PCR

Next, we proceeded with the isolation and analysis of the exosomal RNA cargo. The Total exosome RNA and protein isolation kit, developed specifically for this purpose, uses acid-phenol: Chloroform extraction to provide a robust, initial RNA purification step, followed by a final purification over a glass-fiber filter. Ethanol is added to samples that are passed through a filter cartridge containing the glass-fiber filter, which immobilizes the RNA. The filter is washed, and the RNA is eluted with a low ionic-strength solution.

We followed this protocol to isolate RNA from exosomes derived from HeLa cell culture media and blood serum samples using both the Total exosome isolation reagents and ultracentrifugation procedure[23]. Subsequent analysis with Qubit fluorometer has shown that for exo-

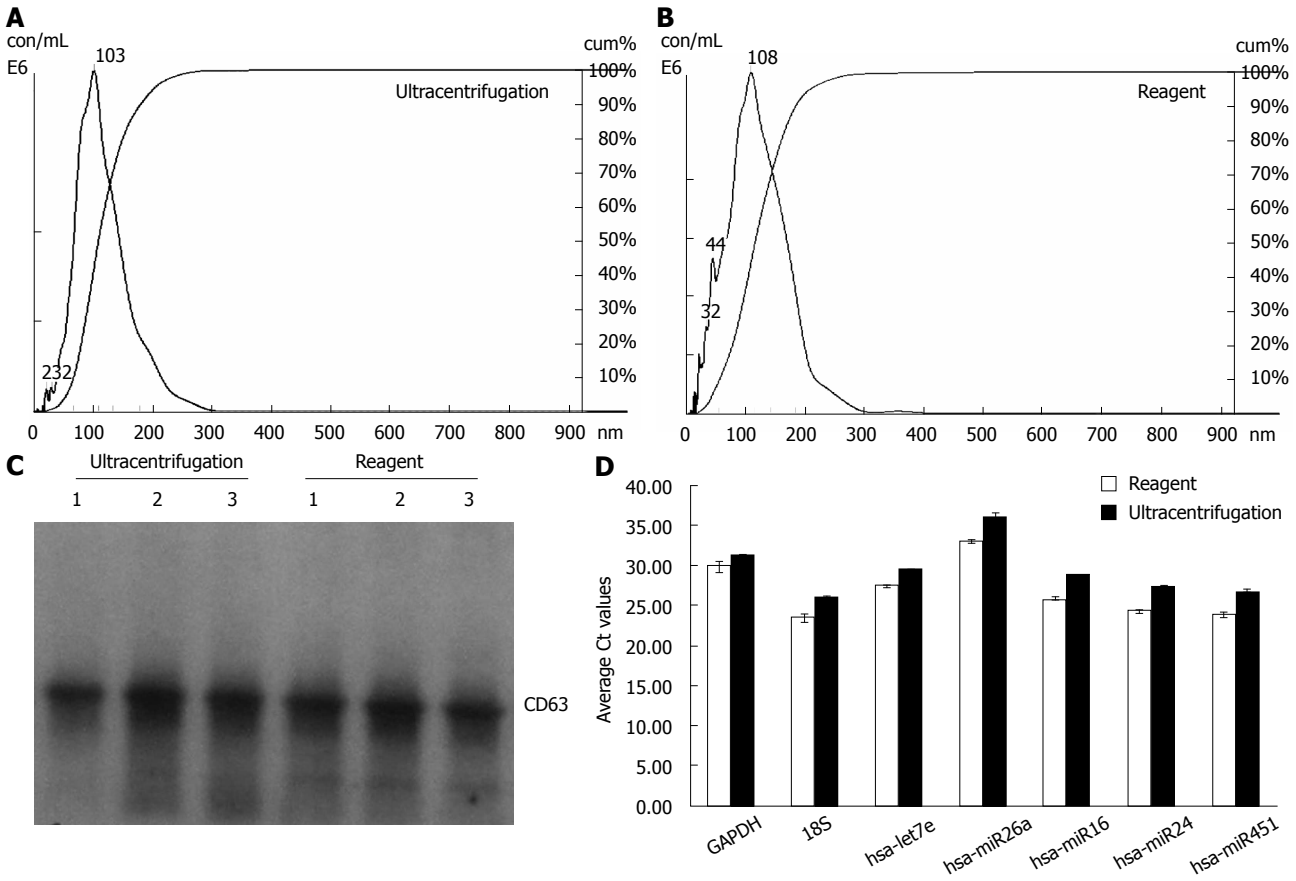

Figure 1 Exosomes isolated from cell media using the reagent are comparable to ultracentrifugation preparations. A, B: Analysis of exosomes recovered from HeLa cell culture media using the Total exosome isolation reagent (from cell culture media) and ultracentrifugation protocol-by Nanosight® LM10 instrument. The profiles are essentially very-finely segmented histograms, indicating the number of particles per mL (in millions) for each size in bins of 1 nm increment from 0 to 1000 nm; C: Western blot analysis for the presence of exosomal marker protein CD63 in cell culture media derived samples. Exosomes from three separate HeLa cell culture media preparations (isolated with either the Total exosome isolation reagent (from cell culture media) or ultracentrifugation) were separated on a Novex 4%-20% Tris-Glycine Gel under denaturing, non-reducing conditions. Standard Western blot procedures with anti-CD63 antibodies were used to detect cell media derived exosomal protein markers; D: Analysis of the exosomal miRNA and mRNA levels in HeLa cell culture media derived samples by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was isolated using the Total exosome RNA and protein isolation kit from exosomes extracted using the Total exosome isolation reagent (from cell culture media) and the ultracentrifugation protocol. Levels of five microRNAs (let7e, miR26a, miR16, miR24 and miR451) and two mRNAs [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S] were quantified by qRT-PCR using TaqMan assays and reagents.

somes isolated from 100 mL of HeLa cell culture media using the ultracentrifugation protocol, it is possible to recover approximately 380 pg RNA. The reagent method resulted in isolation of somewhat more exosomes from the same volume of HeLa cell culture media, containing approximately 520 pg RNA.

RNA recovered from exosomes with the reagent and ultracentrifugation was next characterized by capillary electrophoresis using the Agilent 2100 Bioanalyzer, and a RNA Pico chip. Results are shown in Figure 4. For both types of samples, profiles were similar: the majority of RNA content was small (< 200 nt), but there were some longer RNA species present including full-length 18S and 28S rRNA. This is in agreement with earlier studies[11,12,20], indicating that exosomes primarily contain short RNA (such as miRNA) and degraded mRNA, but also some full-length molecules including mRNA > 1 kb long. Overall, the amount of total RNA recovered, and specifically the small RNA fraction, is higher for the reagent method compared to ultracentrifugation protocol.

Finally, the levels of five microRNAs (let7e, miR26a, miR16, miR24 and miR451) and two mRNAs (GAPDH and 18S) earlier reported to be present in exosomes[11,20], were analyzed by qRT-PCR. Results are displayed in Figure 1D for HeLa cell culture media and Figure 2D for human blood serum. Based on Ct values, 25-33 for the majority of analytes, RNA isolation was efficient and the amount of material recovered is sufficient for standard PCR analysis - RNA recovered from exosomes derived from 3 μL serum or 30 μL cell media is sufficient for one qPCR reaction. The reagent method recovered somewhat higher levels of exosomes-compared to ultracentrifugation procedure, as indicated by 0.5-2 Ct shift up for different RNAs.

DISCUSSION

To conclude, we describe here a novel approach for fast and efficient isolation of exosomes from cell culture media and blood serum, that can be followed by

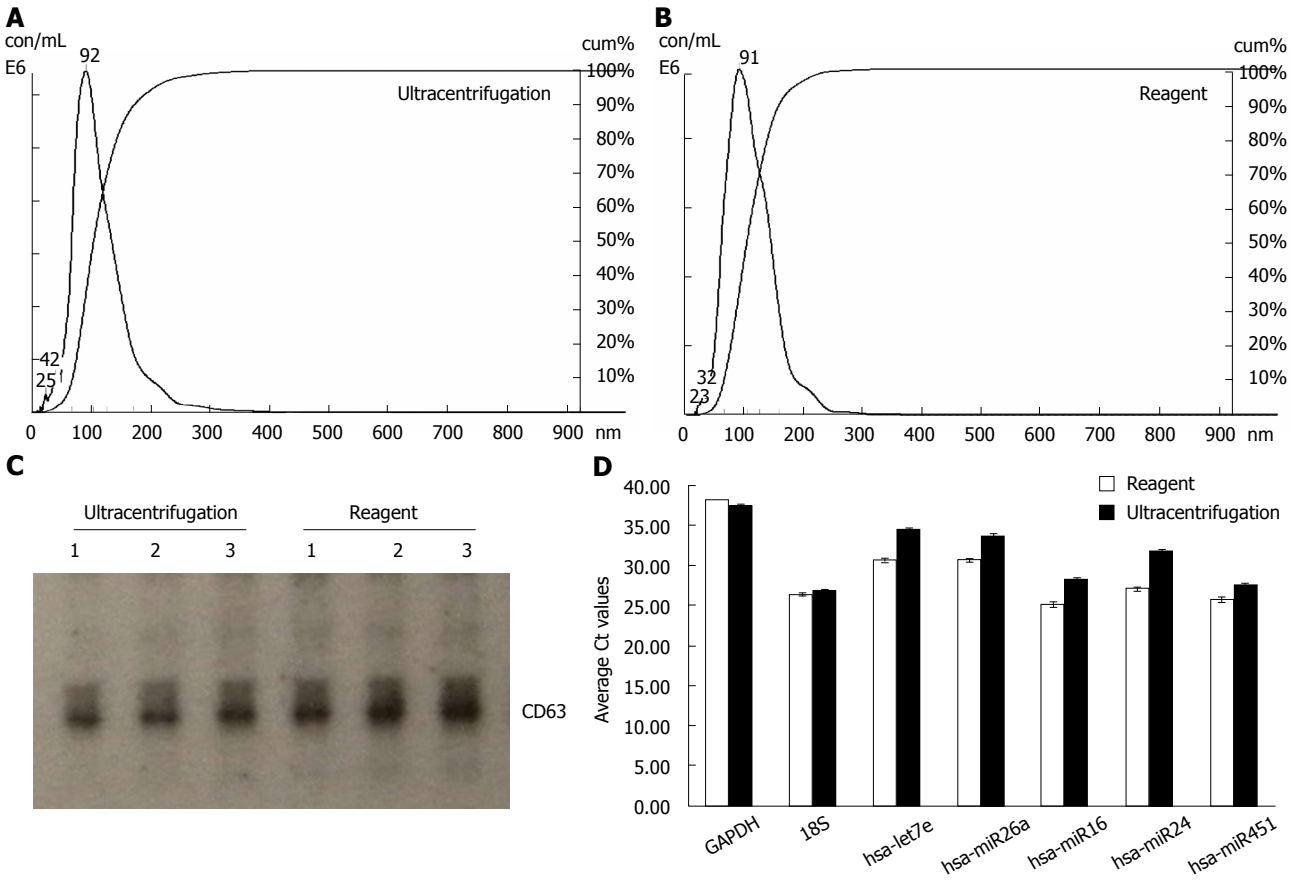

Figure 2 Exosomes isolated from serum using the reagent are comparable to ultracentrifugation preparations. A, B: Analysis of the exosomes recovered from human serum using the Total exosome isolation reagent (from serum) and the ultracentrifugation protocol-by Nanosight® LM10 instrument. The profiles are essentially very-finely segmented histograms, indicating the number of particles per mL (in millions) for each size in bins of 1 nm increment from 0 to 1000 nm; C: Western blot analysis for the presence of exosomal marker protein CD63 in blood serum derived samples. Exosomes from three separate serum preparations (isolated with either the Total exosome isolation reagent (from serum) or ultracentrifugation) were separated on a Novex 4%-20% Tris-Glycine Gel under denaturing, non-reducing conditions. Standard Western blot procedures with anti-CD63 antibodies were used to detect human blood serum derived exosomal protein markers; D: Analysis of the exosomal miRNA and mRNA levels in human blood serum by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). RNA was isolated using the Total exosome RNA and Protein Isolation kit from exosomes extracted using the Total exosome isolation reagent (from serum) and the ultracentrifugation protocol. Levels of five microRNAs (let7e, miR26a, miR16, miR24 and miR451) and two mRNAs [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S] were quantified by qRT-PCR using TaqMan assays and reagents.

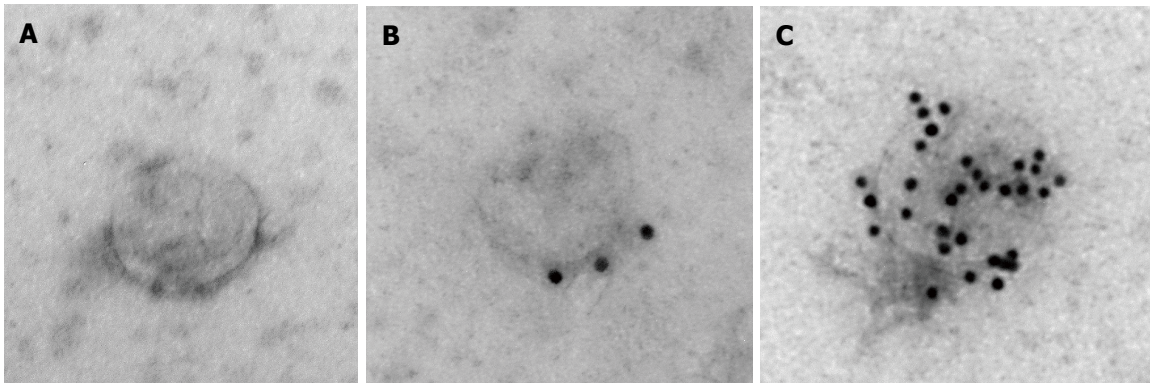

Figure 3 Electron microscopy analysis of exosomes isolated from HeLa cell culture media with the Total exosome isolation reagent. A: Representative image of the unlabeled exosome; B: Exosome immunolabeled with anti-CD63 followed by 10 nm protein A gold nanoparticles; C: Exosome immunolabeled with anti-CD81 followed by 10 nm protein A gold nanoparticles. For immunolabelling, exosome samples were precipitated undiluted at room temperature for 15 min to grids. Next, blocking with 0.5% bovine serum albumin was performed for 10 min. Labeling with anti-CD63 and anti-CD81 antibodies was carried out for 30 min. Following washing steps, Prot A Au were added and incubated for 15 min. After subsequent phosphate-buffered saline and water wash steps, embedding in 0.3% Uranyl acetate in methyl cellulose was finally performed, followed by electron microscop analysis of exosomes.

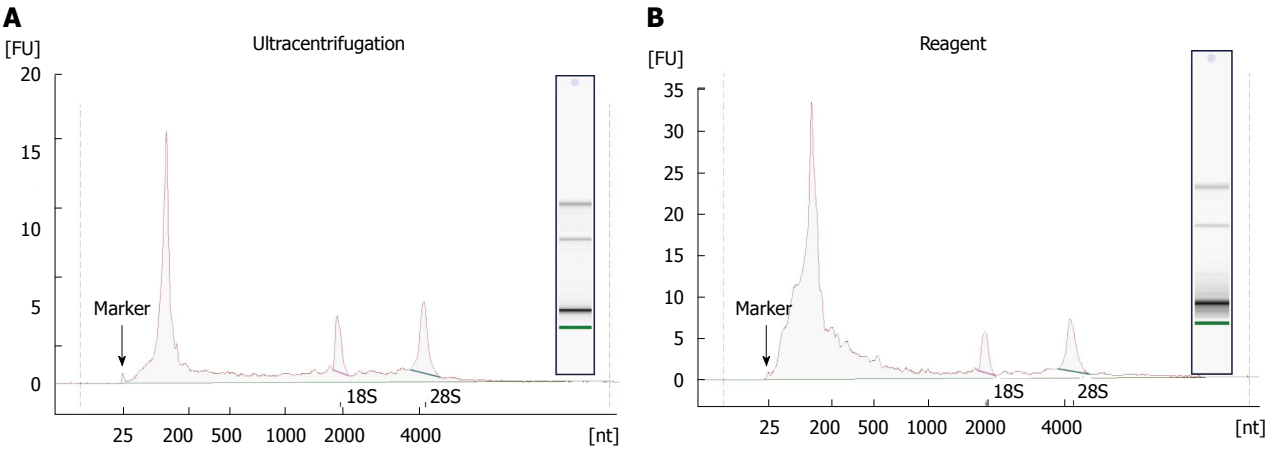

Figure 4 Analysis of RNA cargo of HeLa cell media exosomes by Agilent RNA pico chip. A: Exosomes isolated with ultracentrifugation protocol; B: Exosomes recovered with the Total exosome isolation reagent.

recovery of RNA cargo and its analysis by qRT-PCR or sequencing. The procedure is completed in a fraction of the time, compared to the current standard protocols utilizing ultracentrifugation, and allows recovery of fully intact exosomes in higher yields. This is the first step towards developing standardized techniques and protocols for fast, high throughput and robust isolation of exosomes from various sample types and downstream analysis of their constituents. We believe these reagents and workflows will be highly useful to scientists working on the edge of cellular and molecular biology and focusing on analysis of extracellular (circulating) RNA residing within the exosomes, microvesicles and protein complexes.

COMMENTS

Background

Exosomes are small (30-150 nm) vesicles containing unique RNA and protein cargo, secreted by all cell types in culture. They are also found in abundance in body fluids including blood, saliva, urine. At the moment, the mechanism of exosome formation, the makeup of the cargo, biological pathways and resulting functions are incompletely understood. One of their most intriguing roles is intercellular communication-exosomes function as the messengers, delivering various effector or signaling macromolecules between specific cells. There is an exponentially growing need to dissect structure and the function of exosomes and utilize them for development of minimally invasive diagnostics and therapeutics.

Research frontiers

Several reports have been published to date, using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and next gen sequencing for initial characterization of the RNA content of exosomes derived from severals cell lines, as well as serum, saliva, placenta and breast milk. All these studies utilize ultracentrifugation isolation protocols which allow to produce clean exosome preparations, however, suffer from numerous drawbacks. Critical to further our understanding of exosomes, is the development of reagents, tools and protocols for their simple and robust isolation, characterization and analysis of their RNA and protein contents.

Innovations and breakthroughs

The authors developed the workflow allowing fast and efficient extraction of exosomes, followed by isolation of RNA and its analysis by qRT-PCR. The procedure is completed in a fraction of the time, compared to the current standard protocols utilizing ultracentrifugation, and allows to recover fully intact exosomes in higher yields.

Applications

The workflow presented here allows fast isolation of exosomes and downstream analysis of their constituents, thus enabling basic exosome research as well as development of minimally invasive diagnostic alternative to biopsies.

Peer review

In this study, the authors describe a novel approach for fast and efficient extraction of exosomes from cell culture media and body fluids, and compare the effectiveness of isolation methods with ultracentrifigation protocol. This manuscript is well organized and the experiments are well conducted.

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee.

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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